



If you have discovered material in AURA which is unlawful e.g. breaches copyright, (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please read our [Takedown Policy](#) and [contact the service](#) immediately

**ENGINEERING LIPOSOMAL SYSTEMS TO DEVELOP SOLUBILITY
ENHANCING TECHNOLOGY**

HABIB ALI

Doctor of Philosophy

ASTON UNIVERSITY

AUGUST 2008

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without proper acknowledgement.

/

ENGINEERING LIPOSOMAL SYSTEMS TO DEVELOP SOLUBILITY ENHANCING TECHNOLOGY

Habib Ali
Doctor of Philosophy
2008

This research primarily focused on identifying the formulation parameters which control the efficacy of liposomes as delivery systems to enhance the delivery of poorly soluble drugs.

Preliminary studies focused on the drug loading of ibuprofen within vesicle systems. Initially both liposomal and niosomal formulations were screened for their drug-loading capacity: liposomal systems were shown to offer significantly higher ibuprofen loading and thereafter lipid based systems were further investigated. Given the key role cholesterol is known to play within the stability of bilayer vesicles, the optimum cholesterol content in terms of drug loading and release of poorly soluble drugs was then investigated. From these studies a concentration of 11 total molar % of cholesterol was used as a benchmark for all further formulations.

Investigating the effect of liposome composition on several low solubility drugs, drug loading was shown to be enhanced by adopting longer chain length lipids, cationic lipids and, decreasing drug molecular weight. Drug release was increased by using cationic lipids and lower molecular weight of drug; conversely, a reduction was noted when employing longer chain lipids thus supporting the rationale of longer chain lipids producing more stable liposomes, a theory also supported by results obtained via Langmuir studies- although it was revealed that stability is also dependent on geometric features associated with the lipid chain moiety. Interestingly, reduction in drug loading appeared to be induced when symmetrical phospholipids were substituted for lipids constituting asymmetrical alkyl chain groups thus further highlighting the importance of lipid geometry. Combining a symmetrical lipid with an asymmetrical derivative enhanced encapsulation of a hydrophobic drug while reducing that of another suggesting the importance of drug characteristics.

Phosphatidylcholine liposomes could successfully be prepared (and visualised using transmission electron microscopy) from fatty alcohols therefore offering an alternative liposomal stabiliser to cholesterol. Results obtained revealed that liposomes containing tetradecanol within their formulation shares similar vesicle size, drug encapsulation, surface charge, and toxicity profiles as liposomes formulated with cholesterol, however the tetradecanol preparation appeared to release considerably more drug during stability studies. Langmuir monolayer studies revealed that the condensing influence by tetradecanol is less than compared with cholesterol suggesting that this reduced intercalation by the former could explain why the tetradecanol formulation released more drug compared with cholesterol formulations.

Environmental scanning electron microscopy (ESEM) was used to analyse the morphology and stability of liposomes. These investigations indicated that the presence of drugs within the liposomal bilayer were able to enhance the stability of the bilayers against collapse under reduced hydration conditions. In addition the presence of charged lipids within the formulation under reduced hydration conditions compared with its neutral counterpart. However the applicability of using ESEM as a new method to investigate liposome stability appears less valid than first hoped since the results are often open to varied interpretation and do not provide a robust set of data to support conclusions in some cases.

Key Words *Liposomes, Poorly soluble drugs, Cholesterol, Fatty alcohols, ESEM.*

Acknowledgements

I would like to especially thank my supervisor Professor Yvonne Perrie for her relentless support, supervision and time to offer detailed advice throughout my PhD. Her contribution has been most helpful. My sincere thanks go to my family for their unconditional moral support throughout my research, especially my mum who continually kept me motivated.

I would like to thank Chris Bache and my friend Jiteen Kansara for their invaluable technical support and advice throughout my stressful periods in the laboratory.

I would also like to thank Dr Anil Vangala (Kingston University) for his help with the TEM experiments. Thanks also to Nicola Weston (Nottingham University) for her help with the ESEM studies.

Special thanks to Dr Daniel Kirby for his helpful advice with the Langmuir studies.

I really appreciate the support and constant company I received from my friends in the Drug Delivery Unit, particularly Parveen Madher, Johanna Laru and Tristan Learoyd.

Finally, I gratefully acknowledge the financial support of this research by the Engineering and Physical Sciences Research Council (EPSRC).

List of Publications

Publications resulting from this and related work

H. Ali, A.R. Mohammed and Y. Perrie (2008). The role of liposome bilayer modification on solubility enhancement for propofol. (*In preparation*)

H. Ali, D. Kirby, A.R. Mohammed and Y. Perrie (2008). Fatty alcohols as alternative to cholesterol as liposomal-bilayer stabilisers. (*In preparation*)

Peer-reviewed Abstracts

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2005). The influence of alkyl chain symmetry on liposomal encapsulation of poorly water soluble midazolam. *J. Pharmacy and Pharmacology* **57** (Supplement), p84.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2006). The influence of alkyl chain length on liposomal encapsulation of poorly water soluble drugs. Abstract 1011 33rd Annual International Controlled Release Society symposium, July 22-26, Austria Centre, Vienna, Austria.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2006). The role of a fully charged bilayer on liposomal encapsulation of a poorly water soluble drug. *J. Pharmacy and Pharmacology* **58** (Supplement), p10.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2007). ESEM: An alternative assay for investigating liposome stability. Abstract 422 34th Annual International Controlled Release Society symposium, California, USA.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2007). ESEM: A possible vehicle for assessing liposome stability? *J. Pharmacy and Pharmacology* **59** (Supplement), p13.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2007). Liposome formulations containing an alternative to cholesterol. *J. Pharmacy and Pharmacology* **59** (Supplement), p13.

H. Ali, A.R. Mohammed and Y. Perrie (2008). Effect of hydrophobic drug properties on liposomal loading. 35th Annual International Controlled Release Society symposium, New York, USA.

H. Ali, A.R. Mohammed, D. Kirby and Y. Perrie (2008). Using langmuir monolayer studies to compare tetradecanol and cholesterol as bilayer stabilisers. 35th Annual International Controlled Release Society symposium, New York, USA.

H. Ali, A.R. Mohammed and Y. Perrie (2008). Cholesterol content in liposomal bilayers versus stability. *J. Pharmacy and Pharmacology* **60** (Supplement), p38.

Conference Proceedings

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2006). Investigating fatty alcohols as an alternative liposomal-bilayer stabilizer to cholesterol. International Liposome Conference, London, December 2006.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2007). The effect of anionic and cationic charge on liposomal encapsulation of propofol, a poorly water soluble model drug. United Kingdom and Ireland Controlled Release Society symposium, London, January 2007.

H. Ali, A.R. Mohammed, J. Smith and Y. Perrie (2008). ESEM: a possible vehicle for assessing liposome stability? United Kingdom and Ireland Controlled Release Society symposium, Hertfordshire, January 2008.

Invited Talks

“The influence of alkyl chain symmetry on liposomal encapsulation of poorly water soluble midazolam” presented at the British Pharmaceutical Conference. Sept 26-28 (2005): Manchester: U.K.

List of Contents

	Page
Title Page	1
Summary	2
Acknowledgements	3
List of Publications	4
List of Contents	6
List of Figures	12
List of Tables	18
List of Abbreviations	21
 Chapter One: General introduction	 24
<i>1.0 Introduction</i>	25
<i>1.1 Solubility</i>	25
1.1.1 Poorly water-soluble drugs	26
1.1.2 Physiochemical properties which dictate drug solubility	29
1.1.2.1 pH and pKa	29
1.1.2.2 The partition coefficient (Log P)	29
1.1.3 Approaches to enhancing solubilisation	30
1.1.3.1 Adjusting pH of ionisable drugs	30
1.1.3.2 Co-solvents	32
1.1.3.3 Salt formulations	33
1.1.3.4 Complex formation – cyclodextrins	34
1.1.3.5 Prodrugs	35
1.1.3.6 Carrier systems	37
1.1.3.6.1 Micelles	37
1.1.3.6.2 Emulsions	39
<i>1.2 Multilamellar vesicles</i>	40
1.2.1 Liposomes	40
1.2.1.1 Background and applications	42
1.2.1.2 The classes of liposomes	46
1.2.1.2.1 Multilamellar vesicles	46
1.2.1.2.2 Small unilamellar vesicles	47

1.2.1.2.3 Large unilamellar vesicles	48
1.2.1.3 Properties of liposomes affecting their role as carriers and therefore the solubilisation of hydrophobic drug	49
1.2.1.3.1 Membrane fluidity	49
1.2.1.3.2 Size	51
1.2.1.3.3 Surface charge	51
1.2.2 Niosomes	53
<i>1.3 Method of preparation</i>	54
1.3.1 A method involving the generation of new bilayers: the thin-film technique	54
1.3.2 A method involving physical modification of already created bilayers: the dehydration-rehydration technique	54
<i>1.4 Investigation of liposomal systems using Langmuir monolayers</i>	55
<i>1.5 The model drug candidates</i>	58
1.5.1 Basic drugs	58
1.5.1.1 Diazepam	58
1.5.1.2 Midazolam	59
1.5.2 Acidic drugs	59
1.5.2.1 Ibuprofen	60
1.5.2.2 Phenytoin	60
1.5.2.3 Propofol	61
1.5.2.4 Rifampicin	62
<i>1.6 Lipids used to investigate the solubilisation potential of liposomes</i>	63
1.6.1 Non-ionic surfactants – alkyl esters	63
1.6.2 Phospholipids	65
<i>1.7 Aims and objectives</i>	68
Chapter Two: Materials and methods	69
<i>2.0 Materials and methods</i>	70
<i>2.1 Materials</i>	70
2.1.1 Chemicals	70
2.1.2 Lipids and surfactants	70
<i>2.2 Methods</i>	71

2.2.1 Preparation of multilamellar vesicles (MLV)	71
2.2.2 Preparation of small unilamellar vesicles (SUV)	72
2.2.3 Determination of drug encapsulation efficiency in MLV	72
2.2.4 Determination of encapsulation efficiency using an alternative UV-based method	74
2.2.5 Determination of encapsulation efficiency using HPLC	74
2.2.6 Determination of entrapment efficiency of indomethacin using radio-labelled [^{14}C] indomethacin	75
2.2.7 Preparation of standard curve	75
2.2.8 Determination of vesicle volume distribution	76
2.2.9 Determination of vesicle zeta potential	77
2.2.10 Drug release studies	77
2.2.11 Liposome stability studies	77
2.2.12 Statistical analysis	78
2.2.13 Light microscopy analysis	78
2.2.14 Transmission electron microscopy (TEM) analysis	78
2.2.15 Environmental electron- scanning microscopy (ESEM) analysis	79
2.2.16 Langmuir studies	79
2.2.17 MTT assay	80
2.2.17.1 Resuscitation of frozen COS-7 cell line	80
2.2.17.2 Subculture of COS-7 cells	81
2.2.17.3 Cell quantification	81
2.2.17.4 Cell preparation and plating for in vitro studies	82
2.2.17.5 Samples preparation	82
2.2.17.6 Cytotoxicity assay	82
2.2.18 Red blood cell (RBC) assay	82
2.2.19 Small angle neutron scattering (SANS)	83
2.2.20 Differential scanning calorimetry (DSC)	84

Chapter Three: Solubilisation of drug within bilayer of vesicles: preliminary investigations	85
3.0 Introduction	86
3.1 Determination of drug entrapment within vesicles	88

3.1.1 UV analysis	89
3.1.1.1 Assessment of supernatant	90
3.1.1.2 Assessment of liposomes	90
3.1.2 High performance liquid chromatography analysis	91
3.1.3 Radio-labelling	91
3.2 Drug recovery from centrifugation method	93
3.3 Effect of centrifugation	94
3.4 Lipid and non-ionic surfactant based vesicles	96
3.4.1 Liposomal ibuprofen incorporation	97
3.4.2 Niosomal ibuprofen incorporation	99
3.4.2.1 Non-ionic surfactant vesicles versus phospholipid-based vesicles	100
3.4.2.2 The effect of hydrophobic chain length of non-ionic surfactant	102
3.4.2.2.1 Encapsulation	103
3.4.2.2.2 Vesicle size	104
3.4.2.2.3 Vesicle zeta potential	105
3.5 The effect of liposome morphology on drug loading within liposomes	106
3.6 Conclusions	112
 Chapter Four: The effect of cholesterol within the bilayer constructs	 114
4.0 Introduction	115
4.1 The effect of liposomal cholesterol content on liposomal systems	116
4.1.1 Poorly soluble drug loading	116
4.1.2 Liposome size and surface charge	118
4.2 Vesicle formulation	120
4.3 The effect of liposomal inclusion of cholesterol on bilayer drug release	124
4.3.1 Release Kinetics	127
4.4 Interactions between DSPC and cholesterol in monolayers at the air/water interface	130
4.4.1 DSPC monolayers	132
4.4.2 Cholesterol monolayers	132

4.4.3 Monolayers of mixtures	133
4.5 Conclusions	135

Chapter Five: The role of liposome bilayer composition on drug solubilisation

	137
5.0 Introduction	138
5.1 Studies of liposomes prepared from phosphatidylcholine and its derivatives	139
5.1.1 The influence of lipid alkyl chain length on liposomal encapsulation of poorly water-soluble drugs	140
5.1.2 Temperature effects on encapsulation	144
5.1.3 The influence of lipid alkyl chain length on drug release	145
5.1.4 The effect of phospholipid alkyl chain length on stability	148
5.1.5 The effect of phospholipid alkyl chain length on cell toxicity	152
5.2 The influence of lipid geometry	153
5.2.1 The effect of symmetry of phospholipid on cholesterol optimisation	153
5.2.2 The effect of lipid geometry on liposome entrapment	155
5.2.3 Combination studies	159
5.2.4 The effect of phospholipid symmetry on cell toxicity	161
5.3 The influence of alkyl chain length and symmetry in monolayers at the air/water interface	162
5.4 Effect of drug properties on vesicle characteristics	166
5.5 Charged lipid studies	168
5.5.1 The influence of charged lipids on liposomal-incorporation of propofol	168
5.5.2 The release of propofol from charged liposomes	173
5.5.3 The effect of charge on cell toxicity	176
5.6 Conclusions	179

Chapter Six: Fatty alcohols as alternative to cholesterol as liposomal bilayer stabilisers

	182
6.0 Introduction	183
6.1 The effect of tetradecanol concentration on propofol encapsulation	185
6.2 Investigating alkyl chain length of fatty alcohol	188

6.2.1 The effect of fatty alcohol alkyl chain length on propofol-loading	188
6.2.2 Stability studies	190
6.2.3 Effect of alkyl chain length of stabiliser on drug release	193
6.3 <i>The effect of lipid alkyl chain length used in combination with fatty alcohols</i>	196
6.3.1 Effect on drug loading	196
6.3.2 Effect on drug release	198
6.4 <i>Influence of drug properties on release</i>	199
6.5 <i>Cell toxicity of liposomes containing either cholesterol or tetradecanol</i>	202
6.6 <i>Langmuir monolayer investigations</i>	205
6.7 <i>Conclusions</i>	207
 Chapter Seven: Investigating liposomes using ESEM	 211
7.0 <i>Introduction</i>	212
7.1 <i>The effect of drug-loading on liposome stability</i>	214
7.2 <i>The effect of a near-fully charged bilayer on liposome morphology</i>	218
7.3 <i>Effect of cholesterol concentration on liposome stability</i>	222
7.4 <i>Effect of alkyl chain length on liposome stability</i>	224
7.5 <i>The role of geometry on liposomal morphology</i>	227
7. 6 <i>Conclusions</i>	229
 Chapter Eight: Using small angle neutron scattering to assess bilayer mechanics	 231
8.0 <i>Introduction</i>	232
8.1 <i>Small angle neutron scattering- a brief literature review</i>	233
8.2 <i>The experimental set-up</i>	237
8.3 <i>Results and discussion</i>	239
8.4 <i>Conclusions</i>	240
 Chapter Nine: General discussion and conclusions	 242
9.0 <i>General discussion and conclusions</i>	243
 References	 253

List of Figures

Figure	Description	Page
1.1	<i>A schematic representation of the biopharmaceutical classification.</i>	27
1.2	<i>Structure of a six-glucose α-cyclodextrin.</i>	35
1.3	<i>Schematic representation of the path to the formation of the prodrug fosphenytoin.</i>	36
1.4	<i>The general schematic diagram of micelle formation from amphiphilic molecules.</i>	39
1.5	<i>Liposomes encapsulating drug.</i>	41
1.6	<i>Size and number of bilayers dictating the type of liposome.</i>	48
1.7	<i>Schematic representation of the electric double layer occurring from charged particles in solution.</i>	53
1.8	<i>Structure of diazepam.</i>	59
1.9	<i>Structure of midazolam.</i>	59
1.10	<i>Structure of ibuprofen.</i>	60
1.11	<i>Structure of phenytoin.</i>	61
1.12	<i>Structure of propofol.</i>	62
1.13	<i>Structure of rifampicin.</i>	63
1.14	<i>Structures of various analogues of PC.</i>	66
1.15	<i>Structures of various derivatives of TAP.</i>	67
2.1	<i>Schematic representation of the established 'hand-shaking' method.</i>	72
3.1	<i>The influence of repeat centrifugations on DMPC: cholesterol and DSPC: cholesterol formulations (0.89: 0.11 molar ratio) with midazolam encapsulation at 258nm.</i>	96
3.2	<i>The influence of repeat centrifugations on DMPC: cholesterol and DSPC: cholesterol formulations (0.89: 0.11 molar ratio) with diazepam encapsulation at 228nm.</i>	96
3.3	<i>Sorbitan monoester vesicle forming surfactants.</i>	100

3.4	<i>Sorbitan triester vesicle forming surfactant.</i>	100
3.5	<i>Particle size distribution profile of a liposomal formulation (PC: cholesterol, 0.67: 0.33 molar ratio).</i>	104
3.6	<i>Illustration representing small unilamellar vesicles (between 15nm and 100nm in size) and multilamellar vesicles (between 100nm and 10µm).</i>	107
3.7	<i>A transmission electron micrograph of a drug-entrapped formulation prepared from 16µmol DSPC with 11 total molar % cholesterol content. Image displaying the multiple bilayers of liposomes.</i>	107
3.8	<i>Investigating 'unwashed' MLV encapsulating radio-labelled ¹⁴C indomethacin.</i>	108
3.9	<i>Investigating 'unwashed' SUV encapsulating radio-labelled ¹⁴C indomethacin.</i>	109
3.10	<i>Investigating 'washed' SUV encapsulating radio-labelled ¹⁴C indomethacin.</i>	110
3.11	<i>Schematic illustration of the three gel chromatography processes.</i>	111
4.1	<i>Molecular structure and properties of cholesterol.</i>	116
4.2	<i>The influence of cholesterol content on entrapment.</i>	118
4.3	<i>MLV mean volume distribution of liposomes encapsulating hydrophobic drugs formulated from PC and varying levels of cholesterol inclusion (0-33 total molar %) in the presence of 1.00mg of drug.</i>	119
4.4	<i>A light microscopy image of a PC liposome.</i>	119
4.5	<i>Zeta potential of liposomes encapsulating hydrophobic drugs formulated from PC and varying levels of cholesterol inclusion (0-33 total molar %) in the presence of 1.00mg.</i>	120
4.6	<i>Schematic representation of the lipid film hydration process.</i>	121
4.7	<i>Effect of cholesterol concentration on the release of propofol from liposomes.</i>	129
4.8	<i>The surface pressure-area isotherm of pure monolayer of DSPC at the air/water interface (at 20°C).</i>	132
4.9	<i>The surface pressure-area isotherm of pure monolayer of</i>	133

cholesterol at the air/water interface (at 20°C).

4.10	<i>The surface pressure-area isotherms of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC and cholesterol.</i>	134
5.1	<i>The influence of alkyl chain-length on encapsulation of propofol, midazolam and diazepam.</i>	142
5.2	<i>Diagram proposing the possible increase of hydrophobic region with increasing lipid tail length. (d = diameter).</i>	143
5.3	<i>The effect of phospholipid alkyl chain length on drug release.</i>	146
5.4	<i>Vesicle size of PC- cholesterol, DMPC- cholesterol, DPPC- cholesterol and DSPC- cholesterol formulations encapsulating propofol, stored at 4°C and 25°C, for 28day.</i>	150
5.5	<i>Propofol retention of PC- cholesterol, DMPC- cholesterol, DPPC- cholesterol, and DSPC- cholesterol formulations in aqueous buffer, pH = 7.4, stored at 4°C and 25°C, for 28day.</i>	151
5.6	<i>The effect of alkyl chain length of main phospholipid (DSPC or C₂₄PC) of formulation on mitochondrial dehydrogenase activity of the COS-7 cells as determined by means of the MTT assay after 4hour incubation with liposomes at 37°C.</i>	152
5.7	<i>Structures of symmetrical DMPC (displaying its two C₁₄ alkyl chains) and the asymmetrical MSPC (displaying its alkyl chain moieties of differing length, C₁₄ and C₁₈).</i>	154
5.8	<i>A comparison of midazolam encapsulation by MSPC and PC liposomes prepared using increasing amounts of cholesterol.</i>	155
5.9	<i>The influence of long-chain symmetric and asymmetric lipids on midazolam or propofol encapsulation.</i>	157
5.10	<i>Diagram proposing the possible loss of hydrophobic region in an asymmetric lipid bilayer due to a 'mismatch' interaction between phospholipids possessing two hydrocarbon side-chains of differing length resulting in diameter reduction when compared to a bilayer containing a symmetrical lipid. (d = diameter)</i>	158
5.11	<i>The influence of combining long-chain symmetric and asymmetric lipids on drug encapsulation.</i>	160
5.12	<i>A comparative study of mitochondrial dehydrogenase activity of the COS-7 cells by DSPC and MSPC as determined by means of the MTT assay after 4hour incubation with liposomes at 37°C.</i>	162
5.13	<i>The surface pressure-area isotherms of pure monolayers of DSPC,</i>	165

DPPC, DMPC, MSPC and POPC at the air/water interface (at 20°C).

5.14	<i>The relationship between liposomal (PC: cholesterol, 0.89: 0.11 molar ratio) bilayer encapsulation of drug and lipophilicity or molecular weight of drug.</i>	168
5.15	<i>Encapsulation of propofol by charged liposomes weighed against their neutral phospholipid analogue. The vesicle surface charge of charged vesicles compared against the zeta potential of their neutral counterpart.</i>	171
5.16	<i>Structure of DSPG.</i>	172
5.17	<i>TEM micrographs of liposomes encapsulating propofol (in double-distilled water): DSTAP (0.89: 0.11 molar ratio) vesicles showed a significant reduction in aggregation compared to corresponding neutral DSPC- cholesterol (0.89: 0.11 molar ratio) preparation.</i>	173
5.18	<i>Effect of vesicle surface charge on rate of propofol release.</i>	174
5.19	<i>Effect of alkyl chain length of positively charged TAP series on bilayer release of propofol.</i>	175
5.20	<i>Effect of bilayer charge and lipid concentration on the percentage cell viability of COS-7 cells.</i>	176
5.21	<i>Effect of bilayer charge and lipid concentration on the percent haemolysis of red blood cells.</i>	178
6.1	<i>A transmission electron micrograph of a propofol-entrapped formulation prepared from 16µmol PC containing 11 total molar % tetradecanol content.</i>	187
6.2	<i>Vesicle size of PC, PC: cholesterol, PC: Tet, PC: Hex and PC: Oct formulations encapsulating propofol, stored at 4°C and 25°C, for 28day.</i>	191
6.3	<i>Propofol retention of PC, PC: cholesterol, PC: Tet, PC: Hex and PC: Oct formulations in aqueous buffer, pH = 7.4, stored at 4°C and 25°C, for 28day.</i>	192
6.4	<i>The effect of fatty alcohol alkyl chain length on drug release.</i>	195
6.5	<i>The influence of lipid alkyl chain-length in combination with tetradecanol on drug encapsulation.</i>	197
6.6	<i>The influence of lipid alkyl chain-length in combination with tetradecanol on drug release.</i>	199

6.7	<i>The effect of drug on the release profile of either midazolam, propofol or rifampicin from PC: tetradecanol formulation (0.89: 0.11 molar ratio), in aqueous buffer, under physiological conditions at 37°C.</i>	202
6.8	<i>Mitochondrial dehydrogenase activity of the COS-7 cells determined by means of the MTT assay after 4hour incubation with liposomes, containing cholesterol or tetradecanol, at 37°C.</i>	203
6.9	<i>Effect of liposomes, with inclusion of tetradecanol or cholesterol, on the percent haemolysis of red blood cells.</i>	204
6.10	<i>The surface pressure-area isotherms of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC, tetradecanol and cholesterol.</i>	206
7.1	<i>Schematic diagram of ESEM column, displaying the differential pumping zones.</i>	213
7.2	<i>Environmental scanning electron micrographs of propofol-loaded liposomes (DSPC: cholesterol, 0.89: 0.11 molar ratio) suspended in double-distilled water.</i>	216
7.3	<i>Environmental scanning electron micrographs of drug-free liposomes (DSPC: cholesterol, 0.89: 0.11 molar ratio) suspended in double-distilled water.</i>	217
7.4	<i>Environmental scanning electron micrographs of propofol-encapsulated charged and neutral vesicles suspended in double-distilled water.</i>	220, 221
7.5	<i>Environmental scanning electron micrographs of dry propofol-loaded DSPC lipid films containing 20 total molar % and 33 total molar % cholesterol samples suspended in double-distilled water.</i>	223
7.6	<i>Environmental scanning electron micrographs of dry propofol-loaded lipid films of DMPC: cholesterol and DPPC: cholesterol specimens suspended in double-distilled water.</i>	226
7.7	<i>Environmental scanning electron micrographs of propofol-loaded liposomes formed using POPC and 11 total molar % cholesterol suspended in double-distilled water.</i>	228, 229
8.1	<i>A view of the LOQ sample position. The neutron beam originates from the long silver pipe in the core, having passed through a chamber comprising the collimator and incident beam monitor. A photograph of the Experimental Hall, ISIS, Rutherford Appleton Laboratory, Chilton, Oxford.</i>	234
8.2	<i>A schematic illustration of a small angle neutron scattering</i>	235

experiment showing the scattering behaviour of the source neutrons following interaction with nuclei of test sample.

- 8.3** *Chemical structures of the fully- and partially-deuterated phospholipids (DMPC and DSPC) used for SANS studies.* **238**
- 8.4** *Images displaying preparations placed in meticulously cleaned disc-shaped fused silica cells of path length 2mm.* **240**

List of Tables

Figure	Description	Page
1.1	<i>Presently marketed liposomal formulations</i>	44
2.1	<i>The wavelengths for the drugs studied as determined via wave-scan using a Unicam Helios.</i>	73
3.1	<i>Summary of the characteristics of all model drug candidates investigated.</i>	87
3.2	<i>Summary of drug loading by various methods used to determine entrapment. Encapsulation expressed in terms of 'percent encapsulation' which is typically defined as the total amount of encapsulant encapsulated MLV versus the total initial input of encapsulant.</i>	93
3.3	<i>Summary of drug loading by various methods used to determine entrapment. Results expressed as percent mole per mole (% mol/mol).</i>	93
3.4	<i>Recovery rate of 1.00mg drug dissolved in 30mL (or 60mL) of PBS, following centrifugation at 27,200g for 30min.</i>	94
3.5	<i>Characteristics of liposomes composed of PC and cholesterol with and without the negatively charged DCP.</i>	98
3.6	<i>List of non-ionic sorbitan esters along with their respective HLB (hydrophile-lipophile balance) values.</i>	100
3.7	<i>Characteristics of vesicles composed of PC, cholesterol and 14 total molar % DCP and of vesicles formulated using a sorbitan monoester (Span 60), cholesterol, and 5-14 total molar % DCP.</i>	102
3.8	<i>The effect of alkyl chain length on niosomal ibuprofen loading, MLV size and zeta potential.</i>	105
3.9	<i>A summary of results for liposomal (PC: Cholesterol, 0.89:0.11 molar ratio) encapsulation of ¹⁴C radio-labelled indomethacin comparing 'washed' SUV and MLV against 'un-washed' SUV.</i>	110
4.1	<i>The effect of bilayer composition on hydration of lipid film.</i>	122
4.2	<i>The effect of bilayer composition on hydration of lipid film.</i>	123
4.3	<i>Kinetic values of release of propofol from different liposomal formulations using the correlation coefficient parameter (R²).</i>	128

4.4	<i>The experimental and ideal extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC and cholesterol.</i>	135
5.1	<i>The influence of alkyl chain length of phospholipid on liposome size and charge.</i>	144
5.2	<i>Liposomal encapsulation of diazepam at two different temperatures.</i>	145
5.3	<i>A comparison of the characteristics of MSPC and PC liposomes prepared using increasing amounts of cholesterol.</i>	154
5.4	<i>The influence of asymmetric lipids on mean volume diameter and vesicle charge.</i>	159
5.5	<i>The influence of combining long-chain symmetric and asymmetric lipids on mean volume diameter and vesicle surface charge.</i>	161
5.6	<i>Extrapolated area and collapse pressure of pure DSPC, DPPC, DMPC, MSPC and POPC monolayers at the air-water interface at temperature of 20°C.</i>	166
6.1	<i>Summary of key features of the fatty alcohols.</i>	185
6.2	<i>Characteristics of PC liposomes prepared using increasing amounts of tetradecanol.</i>	186
6.3	<i>The influence of alkyl chain length of fatty alcohol on propofol entrapment, vesicle size and surface charge with comparisons against cholesterol-based preparation.</i>	190
6.4	<i>The influence of lipid alkyl chain-length in combination with tetradecanol on vesicle size and surface charge.</i>	197
6.5	<i>Summary of the characteristics of all model drug candidates investigated.</i>	201
6.6	<i>Summary of results comparing DSPC formulations containing tetradecanol and cholesterol with respect to various parameters.</i>	205
6.7	<i>The experimental and ideal extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC, cholesterol and tetradecanol.</i>	206
7.1	<i>Comparison of characteristics of PC formulations prepared from phospholipids of varying cholesterol content (and 1.00mg</i>	225

propofol).

- 8.1** Formulation (lipid: cholesterol, where applicable, is 0.89: 0.11 molar ratio) description and vesicle size determination for samples analysed via SANS. **239**

List of Abbreviations

ANOVA	Analysis of variance
C ₂₄ PC	Dilignoceroyl phosphatidylcholine
Chol	Cholesterol
CMC	Critical micelle concentration
d	Diameter
DCP	Dicetylphosphate
DIA	Diazepam
DMEM	Dulbecco's Modified Eagle's Medium
DMPC	Dimyristoyl phosphatidylcholine
DMTAP	1,2-dimyristoyl-3-trimethylammonium-propane
DPPC	Dipalmitoyl phosphatidylcholine
DPTAP	1,2-dipalmitoyl-3-trimethylammonium-propane
DSPC	Distearoyl phosphatidylcholine
DSPG	1,2-distearoyl-3-phosphatidylglycerol
DSTAP	1,2-distearoyl-3-trimethylammonium-propane
e.e.	Encapsulation efficiency
ESEM	Environmental scanning electron microscopy
Hex	Hexadecanol
HLB	Hydrophile-lipophile balance
HPLC	High-performance liquid chromatography
FA	Fatty alcohol
g	Relative centrifugal force
IBU	Ibuprofen
LUV	Large unilamellar vesicles

MID	Midazolam
Min	Minutes
MLV	Multilamellar vesicles
Mol	Moles
MSPC	1-myristoyl-2-stearoyl-sn-glycero-3-phosphocholine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
mV	Millivolts
NCE	New chemical entity
nm	Nanometer
Oct	Octadecanol
o/w	Oil-in-water
PBS	Phosphate buffer saline
PC	Egg phosphatidylcholine
PHE	Phenytoin
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PRO	Propofol
RBC	Red blood cell
RIF	Rifampicin
rpm	Revolutions per minute
SANS	Small angle neutron scattering
SEM	Scanning electron microscopy
SD	Standard deviation
Span 20	Sorbitan monolaurate
Span 40	Sorbitan monopalmitate

Span 60	sorbitan monostearate
Span 80	Sorbitan monooleate
Span 85	Sorbitan trioleate
SUV	Small unilamellar vesicles
T _c	Phase-transition temperature
TEM	Transmission electron microscopy
Tet	Tetradecanol
UV	Ultra-violet
w/o	Water-in-oil
ZP	Zeta potential
Π -A	surface pressure- mean molecular area
μ mole	Micro moles

Chapter One:

General introduction

1.0 Introduction

1.1 Solubility

Water is essential to the structure and function of all living matter and it is estimated that the human body is around 60% water by mass. From a pharmaceutical perspective, the behaviour of a new chemical entity (NCE) in water dictates many absorption, movement and elimination issues within the body (e.g. oral absorption and movement through blood), which influence the bioavailability, drug development processes and drug dosage form design (Delaney, 2005).

A growing number of NCE, as well as existing parenteral and orally administered lipophilic drugs, exhibit very low solubility in biological media, this presents a challenge to formulators to prepare delivery systems that deliver drugs at acceptable levels of bioavailability (Fahr *et al.*, 2005; Perrut *et al.*, 2005). Therapeutic application of poorly water-soluble agents is associated with serious problems: low solubility in water is usually affiliated with poor absorption and bioavailability upon oral administration and intravenous administration of aggregates formed by insoluble drug can cause embolisation of blood vessels leading to side effects as serious as failure of the respiratory system. Drug aggregation can also result in high-localised concentrations at the region of deposition associated with local toxicity and/or lowered systemic bioavailability (Lukyanov and Torchilin, 2004).

A past study (Prentis *et al.*, 1999) reported that 41% of failures in novel drug development in seven UK-owned pharmaceutical companies have been as a consequence of poor biopharmaceutical properties, including water insolubility. Most drugs interact via receptors in the body and since this is a molecular interaction, drugs

thus require to be molecularly dispersed, i.e. in solution. Without sufficient aqueous solubility, drugs cannot act, as it will not be present in the body in a sufficiently high concentration in a molecularly dispersed form to have the desired pharmacological effect (Muller and Keck, 2004; Prentis *et al.*, 1999).

The following is a summary of the challenges often faced by the formulation industry when working with many drugs (Muller and Keck, 2004):

- 1) Poor aqueous solubility;
- 2) Inadequate *in vitro* stability;
- 3) Significantly low bioavailability;
- 4) Too short stability *in vivo*;
- 5) Strong toxic side effects;
- 6) Lack of large scale production due to cost etc.

Of these solubility is a key factor (Muller and Keck, 2004).

1.1.1 Poorly water-soluble drugs

There is universal agreement within the pharmaceutical industry that poorly water-soluble drug candidates are becoming more prevalent (Lipinski, 2000; Pouton, 2006). There are presently about 10 000 drug-like substances (Lipinski, 2000) and many of the hydrophobic drug candidates often emerge from contemporary drug discovery programs, presenting formulation scientists with huge technical challenges, such as poor solubility, very low bioavailability, insufficient *in vitro* stability and short *in vivo* stability (Muller and Keck, 2004).

The United States Food and Drug Administration have the Biopharmaceutics Classification System (BCS) guidance (FDA/CDER, 2006) to classify drug substances. This is designed to classify drugs for application to oral delivery; however it is useful in understanding the attribute and requirements of drugs in a wider scale. Drug substances are classified as follows:

Class 1: High permeability, high solubility

Class 2: High permeability, low solubility

Class 3: Low permeability, high solubility

Class 4: Low permeability, low solubility

Following administration, if a class 2 drug can be maintained in a solubilised phase (via formulation design) in the gut lumen, a similar absorption profile to that of a class 1 drug is obtainable; whereas the limited membrane permeability of classes 3 and 4 means little can be achieved through formulation strategies (Pouton, 2006) (Figure 1.1).



Figure 1.1 A schematic representation of the biopharmaceutical classification system suggesting that absorption of class 2 can be enhanced by the way of formulation strategies (Pouton, 2006).

An estimated 40% (50% in case of orally administered drugs) of all newly developed drugs are poorly soluble or insoluble in water (Wong *et al.*, 2006). Many low solubility compounds remain undetected in early scrutiny, as a consequence of overly simplistic techniques utilised to determine solubility in discovery measurements (Avdeef *et al.*, 2008). Since an ever-increasing number of new chemical entities present poor water-solubility, approaches to prevail over this factor are of immense importance in the formulation of drugs. The solubility of a solute depends upon the chemical and physico-chemical properties (e.g. its partitioning coefficient) of the compound and the solvent, as well as such aspects as pressure, temperature, pH of the solution, and to a lesser degree, the status of subdivision of the solute (Martin *et al.*, 1993). Traditional administration routes for drugs are oral delivery or parenteral injection. When administered via the oral route, the drug is introduced into the blood stream from gut through absorption and then diffuses into tissue; whilst in comparison, a parenteral administration results in drug being directly introduced into the blood. By either delivery route, the drug exists in the form of solution. Therefore the drug will distribute *in vivo* according to its physiochemical properties mentioned above (Muller and Keck, 2004).

Many drugs frequently encounter a range from physical dilemmas and chemical problems to difficulties associated with initial administration and large-scale industrial production. Specific problems include poor solubility, insufficient *in vitro* stability, poor *in vivo* stability, low bioavailability, and strong side-effects (Muller and Keck, 2004). For example, the cytotoxic alkaloid camptothecin shows anti-tumour activity in numerous animal tumour models. However, the drug suffers from adverse water solubility, and several toxic effects (including anaemia, neutropenia, and skin

rash). Since it is essential for drug molecules to be dissolved if they are to interact at the molecular level with targets such as receptors, issues surrounding drug solubility have vital implications for novel drug development.

1.1.2 Physicochemical properties which dictate drug solubility

The physicochemical properties of drugs both in solution and their respective solid state play a vital role in drug formulation. If a drug is administered in solution, it must be adequately soluble. The hydrophobicity/hydrophilicity of the drug influence solubility as well as pH of solution (Barich *et al.*, 2005).

1.1.2.1 pH and pKa

The majority of drugs used in medicine are, weak acids, weak bases or both (Cairns, 2003; Kim, 2004) and the extent of these is dependent on the pH of the dissolving media (Kim, 2004). The pKa values, express the strength of acids and bases, where $pK_a = -\log_{10}K_a$. It gives the value at which 50% of the drug is in the ionised form, with a decreasing value of pKa representing ascending strength of the acid (Cairns, 2003). Overall, acidic drugs are less soluble in solutions at low pH (when they are primarily unionised and in their molecular form) whilst soluble in more polar solvents at high pH values (when they are chiefly ionised). Whilst in comparison, bases are less soluble when the pH is elevated (as the base is unionised) and tend to be water-soluble at low pH (Cairns, 2003).

1.1.2.2 The partition coefficient (Log P)

When a substance is added to two immiscible solvents, it will tend to dispense itself between the pair of solvents according to its affinity for each phase. A compound of a

polar nature (such as an ionised drug) will be inclined to favour the polar or the aqueous phase, while a non-polar structure (e.g. an unionised drug) will favour the non-polar or organic phase. This distribution according to affinity is called the partition coefficient (P) of the compound, and may be expressed as $P = \text{organic/aqueous}$ (Cairns, 2003). Lipophilicity is expressed by $\log P$, the logarithm of the octanol-water partition coefficient. It is a key physico-chemical parameter, as it is used as a measure of the hydrophobicity of compounds (Momo *et al.*, 2005) and is used to predict the absorption, distribution and elimination of drugs within the body (Lipinski, 2000).

$\log P$ is of particular significance in drug design as poor solubility and poor permeability are among the chief culprits for failure during drug development. It is therefore important to determine these physicochemical properties associated with the drug. This can be calculated either from experiments or theoretically from approximate empirical formula; however these have limited reliability (Khadikar *et al.*, 2002; Momo *et al.*, 2002; Remko and von der Lieth, 2004).

1.1.3 Approaches to enhancing solubilisation

Various approaches can be undertaken in order to improve drug solubility. Such processes include adjusting the pH of ionisable drugs, solubilisation by e.g. co-solvents, complex formation using certain complexing agents, chemical modification of a drug resulting in the formation of prodrugs, and utilising various carrier systems, such as liposomes and micelles.

1.1.3.1 Adjusting pH of ionisable drugs

One of the key factors responsible for solubility and dissolution of a compound is its ability to undergo dissociation into ionic species, which in turn is dependent on the pH (represented by equation 1.1) of the medium intended for its dissolution (Seedher and Bhatia, 2003).

The pH (hydrogen ion potential) of a solution is defined as:

$$\text{pH} = -\log_{10} (\text{H}^+) \quad (1.1)$$

Where, (H^+) is the hydrogen ion concentration.

Majority of drugs are ionised in aqueous media, and can therefore exist in a neutral or a charged form. Solubility of drug in aqueous conditions, consequently, is a function of the nature of the aqueous medium and particular drugs display aqueous solubility below only some of the physiological pH conditions. The most notable example of this is within the gastro-intestinal tract where the pH ranges from 1-2 in the stomach, increasing to 7-8 or even higher in the intestine (Kobayashi *et al.*, 2001). Such drastic changes in pH will influence the solubility of drugs (whether acidic or basic). One such example is carvedilol which is soluble in the stomach's acidic condition, whereas it is practically insoluble in the neutral-slightly acidic environment of the intestine (Perrett and Venkatesh, 2006).

Similarly, midazolam shows good solubility in low pH but decreased solubility in physiological conditions. Midazolam belongs to a family of compounds known as imidazobenzodiazepines. The compounds harbour a unique structure which allows for their physiochemical characteristics. The imidazole ring constitutes of a nitrogen atom, which renders the ring basic with a pKa value of around 6. In an acidic pH of

less than 4, the benzepine ring of midazolam is open, leading to an enhanced water solubility of the drug. This has allowed it to be packaged without diluents such as propylene glycol, resulting in the reduction of veous irritation and, potentially, dysrhythmias. Whilst in contrast, in physiological pH, the ring closes, and midazolam becomes significantly lipophilic (Andersin and Tammilehto, 1995).

In comparison, there are certain drugs that do not comprise of any ionisable moiety and therefore cannot be ionised at any pH. An example is rofecoxib, which does not harbour any ionisable group as a result making it virtually impassive to pH moderation (Seedher and Bhatia, 2003).

1.1.3.2 Co-solvents

The addition of a co-solvent to a formulation is a commonly used process for enhancing the solubility of a drug (Seedher and Bhatia, 2003; Nandi *et al.*, 2003). Polyethylene glycol 400 (PEG-400) is a commonly used co-solvent for enhancing the aqueous solubility of sparingly soluble drugs (Nandi *et al.*, 2003). The physical and chemical properties of the solvent such as polarity of solvent and the potential of the solvent to hydrogen bond with the drug moieties are the major contributory factors in the dissolution of drugs by pure solvents (Seedhar and Bhatia, 2003). Previous investigations by Seedhar and Bhatia (2003) examined the solubility of celecoxib, rofecoxib, meloxicam, and nimesulide (cox-2 inhibitors) in various non-aqueous solvents (i.e. alcohols, glycols, and PEG-400). The solubility of celocoxib was exceptionally high in all solvents considered. For all the drugs, solubility was exceptionally high in polyethylene glycol, while solubility (of each drug) decreased with increasing chain length of the alcohols. Furthermore, the solubility of celecoxib,

rofecoxib, and nimesulide could be enhanced significantly by using ethanol and PEG-400 mixtures.

However, the use of co-solvents can have its drawbacks. For example, sodium phenytoin formulation for parenteral administration is a very alkaline solution containing 10% ethanol and 40% propylene glycol. Local irritation has been reported as a result of intravenous delivery (Stella, 1996). Another shortcoming of co-solvents is the danger of drug precipitation upon dilution of solubilised drug with aqueous solutions (e.g. physiological fluids upon a parenteral administration) (Lukyanov and Torchilin, 2004).

1.1.3.3 Salt formulations

Formulation of drugs as salts instead of the use of the drug in its acid or base form is the most commonly used method to improve aqueous solubility (O'Connor and Corrigan, 2001; Serajuddin, 2007). The aqueous solubility of an acidic or basic drug as a function of pH dictates whether the compound will form suitable salts or not. If salts are generated, the ease at which they would dissociate back into their free acid or base forms will depend on interrelationships of several factors, such as S_0 (intrinsic solubility), pH, pK_a , K_{sp} (solubility product) and pH_{max} (pH of maximum solubility) (Dressman, 2007; Serajuddin, 2007).

Whilst it has been shown that in many cases the solubility of a salt is increased compared to the free acid or base form of the drug, the extent of this increase however, depends on the type of salt formed. O'Connor and Corrigan (2001) studied the physicochemical properties of three diclofenac salts, diclofenac sodium (DNa),

diclofenac *N*-(2-hydroxyethyl)pyrrolidine (DHEP) and diclofenac diethylamine (DDEA), and their different solid state forms to determine the influence of salt form on solubility; the equilibrium solubility (at 25°C) followed the trend of DDEA (33mM) < DNa (66mM) < DHEP (273mM). Additionally, the monohydrate form of DHEP was found to be 1.8 times less soluble compared to the anhydrate form, while DDEA anhydrate was approximately 1.7 times as soluble as the monohydrate form. In another instance, Streng *et al.* (1984) reported that solubilities of terfenadine salts formed with phosphoric acid, hydrochloric acid, methanesulfonic acid and lactic acid showed up to 10-fold differences, ranging from 0.5mg/mL to 5mg/mL.

1.1.3.4 Complex formation – cyclodextrins

Cyclodextrins have been frequently used in the pharmaceutical industry as solubilising agents. They are a family of cyclic oligosaccharides which can form inclusion complexes with large organic molecules, thus improving their dissolution rate. Cyclodextrins (Figure 1.2) have a truncated cone with a hydrophilic outer surface containing a somewhat lipophilic central cavity into which a drug can be enclosed to yield a non-covalent bonded inclusion complex either in an aqueous solution or in a solid phase. This complex-forming process is comparable to a molecular encapsulation. In aqueous solutions, the complex formation occurs by a process in which water molecules located inside the crater are displaced by the entire drug molecule or some lipophilic structure of the molecule, thus lowering the energy of the system (Rodier *et al.*, 2005).

Cyclodextrins have been utilised to enhance the stability and solubility of a range of poorly water-soluble drugs e.g. miconazole (Barillaro *et al.*, 2004) and progesterone

(Nandi *et al.*, 2003). The most widely utilised are the α -, β - and γ - cyclodextrins, generated by six, seven and eight glucose units, respectively (Rodier *et al.*, 2005). The number of elementary glucose units determines the cavity size of the cyclodextrin (Georg *et al.*, 2005).

The most common stoichiometry of drug/cyclodextrin complexes is in a 1:1 ratio, i.e. one drug molecule gives a complex with one cyclodextrin molecule (Loftsson *et al.*, 2005). The main drawbacks of using cyclodextrins to improve drug solubility include elevated production costs and toxicity (Barillaro *et al.*, 2004).



Figure 1.2 Structure of a six-glucose α -cyclodextrin and definition of the atomic labels (Georg *et al.*, 2005).

1.1.3.5 Prodrugs

An increasing percentage of new drug approvals in fact contain prodrugs (Dressman, 2007) with approximately 5–7% of the drugs approved worldwide classified as prodrugs (Rautio *et al.*, 2008). Prodrugs are compounds which have to undergo biotransformation before exhibiting a biological response. By the addition of

functional groups that increase solubility to the drug molecule prodrugs with enhanced solubility can be prepared. The additional groups themselves are not pharmacologically active parts of the molecule and must be removed by the action of enzymes or through chemical reactions to regenerate the biologically active drug molecule from the prodrug (Rautio *et al.*, 2008; Stella, 1996).



Figure 1.3 *Schematic representation of the path to the formation of the prodrug fosphenytoin (Stella, 1996).*

Stella (1996) reviewed the development of fosphenytoin (route of synthesis shown in Figure 1.3), a parenterally useful prodrug form of the sparingly soluble agent, phenytoin. Phenytoin is parenterally administered as sodium phenytoin, formulated as a highly alkaline solution (pH = 12) consisting of 10% ethanol and 40% propylene glycol. Intramuscular administration of the drug results in erratic system delivery due to probable crystallisation of phenytoin at the site of delivery as a result of co-solvent dilution and pH adjustment. Furthermore, when delivering the phenytoin formulation intravenously, the formulation must be administered undiluted (or minimally diluted) over a prolonged period of time due to acute safety purposes (e.g. local irritation).

Stella (1996) also stated that when sodium phenytoin was intravenously administered to dogs, autopsy results showed phenytoin precipitation in the plasma resulting in lung entrapment.

The phenytoin prodrug, fosphenytoin, has an aqueous solubility of 142mg/mL, a value equivalent to 88.2mg/mL of its parent drug at physiological pH (phenytoin has a solubility of 20-25µg/mL at neutral pH values) (Stella, 1996).

1.1.3.6 Carrier systems

Colloidal carrier systems have been found to be efficient drug delivery vehicles due to their diversity in structure function *in vivo* and *in vitro*. Various types of these systems exist, including micelles, emulsions, and multilamellar vesicles (niosomes and liposomes) (Sinha *et al.*, 2002).

1.1.3.6.1 Micelles

Surfactants are compounds, whose molecules are amphiphilic, i.e., they feature both lipophilic and hydrophilic moieties. The aggregation of surfactants or surface-active agents in an aqueous solution results in the formation of micelles (Figure 1.4) when a certain minimum concentration (called the critical micelle concentration, CMC) is reached (Di Profio, 2005). However not all surface-active agents form micelles and the critical packing parameter (CPP; equation 1.2), at depicts the predisposition of an amphiphile to form aggregates, whether micellar or vesicular.

The CPP has the following form:

$$CPP = v/I_c A_0 \quad (1.2)$$

Where,

V = the hydrocarbon chain volume

l_c = the critical lipophilic chain length (the length beyond which hydrocarbon chain fluidity may longer be certain)

A_0 = the hydrophilic head group area.

A calculated value of $CPP < 0.5$ signifies formation of the micelle, figures between 0.5 and 1.0 indicating that the surfactant structures assemble preferentially into vesicles, whereas a $CPP > 1.0$ would prompt a surfactant monomer to transform into inverted micelles (Florence and Attwood, 1998).

Appropriate choice of surfactants can allow for the formulation of micelles that are chemically stable, optically transparent, relatively non-toxic and effective in solubilising poorly soluble drugs solutes/reactants (Lin and Yamada, 2003; Torchilin, 2001). The low-molecular-weight pharmaceutical surfactants utilised for micelle production (e.g. Tween 80 and Tween 85; Cortesi and Nastruzzi, 1999) display low toxicity and high solubilisation power towards poorly soluble agents. Being in a micellar form, the solubilised drug is sufficiently shielded from potential inactivation induced by its biological surroundings (Torchilin, 2001). The relatively small size coupled with their dynamic features, suggest micelles have the potential to develop rapid and highly effective methodologies for solubilising analytes in aqueous media (Saitoh *et al.*, 2005). However, micelles made of such surfactants usually boast relatively high CMC and are unstable upon dilution (for example, with the blood volume upon intravenous administration) (Torchilin, 2001).

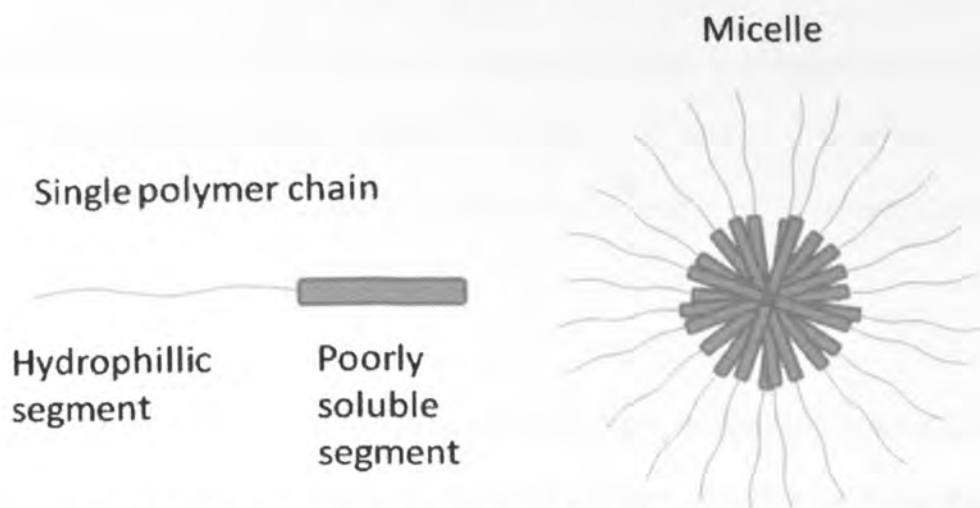


Figure 1.4 The general schematic diagram of micelle formation from amphiphilic molecules (modified from Torchilin, 2001).

Cortesi *et al.* (1997) report that the solubility of the potent cytotoxic drug amphotecin in a micellar solution (a 1:1 mixture of two surfactants, Tween 80 and Tween 85), was five-times higher than the solubility of the drug in water. Additionally, *in vitro* analysis on human erythroleukaemic K562 cells revealed a similar or slightly improved effect when compared with that displayed by the free drug, at the same concentrations.

1.1.3.6.2 Emulsions

An emulsion is a dispersion of two or more immiscible liquids stabilised by a surfactant or an emulsifier coating the droplets and prohibiting coalescence via the reduction of interfacial tension or generating a physical repulsion effect between the droplets (Florence and Attwood, 1998; Stevens *et al.*, 2003). Predominantly, there are two types of emulsions utilised in parenteral drug delivery systems:

- a) Water-in-oil (w/o) emulsions. Used in sustained delivery of steroids and vaccines via intramuscular injection;

- b) Oil-in-water (o/w) (or lipid) emulsions can undergo administration by way of a number of parenteral routes including subcutaneous and intramuscular. However, they are chiefly injected intravenously in parenteral nutrition applications.

Chemical and physical stability of the constituents used in emulsions is a necessity, and they must be able to be sterilised and endotoxin-free. However emulsions whilst kinetically stable, depending on their globule size are often thermodynamically unstable, and unless prepared as microemulsions they are less stable than the micellar system (Cortesi and Nastruzzi, 1999).

Neoral[®] is a self-emulsifying drug delivery system containing the active ingredient cyclosporine (a calcineurin inhibitor) currently available on the market (Abou-Jaoude *et al.*, 2005). However, despite its success in controlling transplantation rejection, use of neoral to minimise rejection after a transplantation procedure is often associated with unnecessary side-effects, which requires switching to safer immuno-suppressants, including other calcineurin inhibitors, e.g. tacrolimus (Abou-Jaoude *et al.*, 2005).

1.2 Multilamellar vesicles

1.2.1 Liposomes

Liposomes are microscopic spherical vesicles (Figure 1.5) of highly ordered lipid molecules which are spontaneously generated when phospholipids undergo hydration (Talsma *et al.*, 1992), arranging themselves in sheets, the molecules aligning side-by-side in 'like' orientation ("heads" up and "tails" down). These sheets then connect

tail-to-tail to form a bilayer membrane, which encloses some of the water in a phospholipid sphere (Kirby and Gregoriadis, 1999). The liposomal characteristics depend on the manufacturing protocol and choice of bilayer components. The sizes of the structures can range from around 30 nanometers (nm) to the micrometer (μm) size range, and can either be unilamellar (only one bilayer surrounding an aqueous core) or multilamellar (several bilayers concentrically oriented around an aqueous core) (Uchegbu, 1999). The bilayers can be in a rigid or fluid state at ambient temperature (T_a). The fluid phase is obtained with amphiphiles with a gel-to-liquid temperature (T_c , the temperature at which melting of fatty acid chain occurs) below the T_a , whilst the rigid state requires amphiphiles with a T_c higher than T_a (Gregoriadis *et al.*, 1999). Above the main gel-liquid transition temperature (T_c) of the phospholipids, when dispersed in an excess of water, they vesiculate spontaneously and enclose the aqueous core (Ebrahim *et al.*, 2005; Felnerova *et al.*, 2004).

As a result of their aqueous inner compartment, liposomes are significantly suited for transporting aqueous drugs. However, their residue structure can also carry poorly soluble drugs such as taxol by incorporating them in the membrane as opposed to the aqueous core (Shieh *et al.*, 1997) (Figure 1.5).

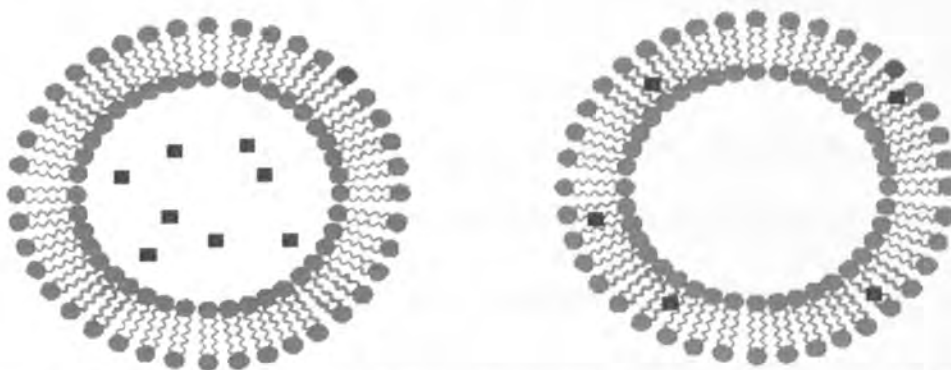


Figure 1.5 Liposomes encapsulating drug (■) - (left) aqueous soluble drug encapsulated in aqueous compartment; (right) hydrophobic drug in the liposome bilayer (modified from Uchegbu, 1999).

1.2.1.1 Background and applications

Subsequent to the first description of liposomes in the mid-60s (by Bangham *et al.*, 1965) the use of liposomes as carriers for selective delivery of drugs has received considerable attention since their first use of such a system as an immunological adjuvant for the purposes of human and veterinary vaccines containing viral or bacterial vaccines (Allison and Gregoriadis, 1974) and when they were first proposed as drug carriers in cancer chemotherapy (Gregoriadis *et al.*, 1971; Gregoriadis *et al.*, 1974). These vesicles have been utilised for the treatment of various diseases, including cancer and auto-immune disorders (Felnerova, *et al.*, 2004) and several commercial products are available (Table 1.1).

Key abilities of liposomes include their ability to reduce the toxic effects and increase the bioavailability of encapsulated drugs. This is due to their particulate nature. Particles which enter the systemic circulation are taken up by the reticuloendothelial system (RES) (Fresta *et al.*, 1995). The RES, which is also referred to as the mononuclear phagocyte system (MPS), plays a crucial role in the body's defence system as it is responsible for filtering out and destroying bacteria, viruses and foreign substances, and destroying dead and abnormal cells/tissue. The RES broadly refers to a group of organs and tissues that contain phagocytic cells, including spleen, liver, lymph nodes and lymphoid tissue (Gregoriadis, 1985). The main function of these phagocytic cells is to eliminate and remove foreign material, including bacteria and proteins. Therefore when injected intravenously, liposomes are cleared rapidly due to the action of the liver and spleen macrophages and this site-specific, but passive, mechanism of clearance is a feature of the immune system (Kirby and Gregoriadis, 1999). For example, a study by Deol and Khuller (1997) formulated liposomes with

improved affinity towards lung tissue by the way of modifying the surface of stealth vesicles by tagging O-stearylmylopectin (O-SAP). Liposomes consisting of egg phosphatidylcholine, cholesterol, dicetylphosphate, O-SAP and monosialogangliosides/distearylphosphatidyl-ethanolamine-poly(ethylene glycol) 2000 were identified to be the most stable in serum. The liposomal tissue distribution revealed an enhanced accumulation in lungs when compared to reticuloendothelial systems of normal and mice infected with TB. *In vivo* stability of these vesicles showed the slow and managed release of their entrapped constituents. The rifampicin-loaded liposomes demonstrated less toxicity towards peritoneal macrophages and reduced *in vivo* toxicity as compared to the free drug. The team of scientists concluded that their results indicate liposomes to be an improved drug delivery system for experimental TB.

Liposomal formulations have been suggested as potential tools for improved therapeutic efficacy in several disease states. For instance, although presently there are various anticancer drugs that are highly cytotoxic to tumour cells *in vitro*, the lack of selective anti-neoplastic impact *in vivo* limits their clinical use. One of the chief drawbacks of anti-tumour agents resides in their low therapeutic index (i.e. the differential between effective and toxic concentrations is low) (Sharma and Sharma, 1997). Thus, there is a requirement for effective delivery vehicles that not only behave as a formulation aid but change the drug bio-distribution in a way that improves the therapeutic index (Bellot *et al.*, 2001; Kirby and Gregoriadis, 1983).

The rationale for using liposomal delivery for cancer therapeutics is that liposomal vesicles are known to target tumour sites due to the Enhanced Permeability and

Retention (EPR) effect- the preferential accumulation of polymeric nano-sized drug carriers (such as liposomes) at tumour sites as opposed to normal tissues (Kim *et al.*, 2008; Matsamura and Maeda, 1986). This is caused by the disorganised vascularisation and defective vascular architecture of tumours (Kim *et al.*, 2008).

Considering liposomes have been studied as drug carrying vehicles since the early 1970's, it took some time (nearly 2 decades) to develop the initial products for application (Tiberg, 2003). The first liposomes appeared in the market in 1986 in the cosmetic formulation "Capture" by Dior. However, it wasn't until the early nineties when it made its maiden entrance as pharmaceutical products (Alveofact[®] and Ambisome[®]) (Muller and Kerk, 2004). With the development of the liposome technology, several other marketed products and clinical trials have been commissioned, for instance, the liposomal preparation of amphotericin B, Ambisome[®] (Table 1.1).

Brand name	Generic name	Indication
Abelcet	Amphoterecin B	Antifungal
Ambisome	Amphoterecin B	Antifungal
DaunoXome	Daunorubicin	Kaposi's Sarcoma
Definity	Octafluoropropane	Imaging
DepoCyt	Cytarabine	Oncology
DepoDur	Morphine	Pain
Doxil	Doxorubicin	Kaposi's Sarcoma
Epaxal	Vaccine	Hepatitis A
Inflexal V	Vaccine	Influenza
Myocet	Doxorubicin	Metastatic breast cancer
Visudyne	Verteporfin	Age-related macular degeneration

Table 1.1 Presently marketed liposomal formulations (adapted from Ebrahim *et al.*, 2005 and Reimer *et al.*, 2005).

Two different formulations of liposomal anthracyclines have been approved for the treatment of acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma (Bellot *et al.*, 2001): Caelyx (R) (US tradename of DOXIL[®]), a liposomal formulation of doxorubicin manufactured by Sequus Pharmaceuticals was approved by the United

States in 1995 (Liu *et al.*, 2001); DaunoXome is a liposomal formulation of daunorubicin. Liposomal preparations were proposed several years ago as a possible way for reducing the toxicity of these anthracyclines, especially their cardiotoxicity (Bellott *et al.*, 2001). Both formulations have displayed a significant activity in this pathology and have been under investigation in other solid tumours and haematological malignancies (Massing and Fuxius, 2000).

Liposomes are also being investigated as carriers for other routes such as topical and transdermal delivery for drugs such as steroids, local anaesthetics and anti-microbial agents (Biju *et al.*, 2006). For example, Manosroi *et al.* (2005) list several drugs such as estradiol, dithranol, lorazepam, ketoprofen, amphotericin B and tranexamic that have successfully undergone MLV encapsulation for the purposes of topical administration.

In ophthalmology, drug-loaded liposomes have also displayed several advantages for administration to anterior and posterior segments. In the anterior segment, which includes front third regions of the eye (i.e. cornea, iris and ciliary body), liposomes have been utilised in diagnostic and therapeutic routes for surface abnormalities, such as keratitis and uveitis. In the posterior segment which includes the back two-thirds of the eye (i.e. retina, choroids and optic nerve), liposomal application has involved subconjunctival and intravitreal injection, as well as insertion of depot devices. This has revealed prolonged clearance rates and inferior toxicity (Ebrahim *et al.*, 2005).

Recently, liposomes have entered previously untouched fields. For instance, in the last decade, it has been proposed that liposomes may be a suitable way of delivery of

DNA to antigen presenting cells as it is recognised that locally injected liposomes are keenly taken up antigen presenting cells penetrating the site of administration or in the lymphatics. There is also the added bonus of protection the vesicles will provide to the DNA moiety from specific enzyme attack (Gregoriadis *et al.*, 2000).

In a number of diseases, e.g., cancer, fungal infections and leishmaniasis, liposomes have been suggested as effective drug carrier systems with minimal complications (Deol *et al.*, 1997; Vyas *et al.*, 2004). However, despite having quite a number of products on the market (Table 1.1) (as well as a significant number in the clinical phases), the overall results are far behind the euphoric anticipation of the 1980s, with pharmacological limitations, relative high costs and physical stability issues being blamed as important drawbacks (Massing and Fuxias, 2000).

1.2.1.2 The classes of liposomes

Liposomes can be characterised by their size (related to number of lamellae) as well as shape, with the former being the most common index (Liu *et al.*, 2001). Small unilamellar vesicles (SUV), multilamellar vesicles (MLV) and large unilamellar vesicles (LUV) are the three chief morphological classes of liposomes which can be further subdivided (e.g. oligolamellar which lies in between LUV and MLV) (Kirby and Gregoriadis, 1999) (Figure 1.6).

1.2.1.2.1 Multilamellar vesicles

MLV consist of a population of vesicles spanning a broad range of sizes (100-10,000nm) and comprising of internal volumes of between 0.5 and 1.0 μ L/ μ mol lipid (Ahl *et al.*, 1994); each vesicle is typified by the presence of multiple lamellae (i.e.

bilayers). Multilamellarity carries the benefit of reducing the rate of loss of diffusible incorporated solutes in the presence of plasma (Kirby and Gregoriadis, 1984). MLV are also simple to manufacture and encompass a structure that is relatively rugged (Kirby and Gregoriadis, 1999). However, their large size spectrum is regarded as a distinct disadvantage for many medical applications necessitating administration via the parenteral route, as it results in rapid clearance from the blood-stream by the cells of the reticuloendothelial system although this mechanism has been utilised for passive targeting of substances to the fixed macrophages pertaining to the liver and the spleen such as the delivery of antimicrobial agents to microorganisms which normally deflect therapy as a result of their localisation within these cells (Kirby and Gregoriadis, 1999). The heterogeneous population of vesicles can be sonicated or extruded via polycarbonate filters to yield small, more uniformly dimensioned populace of SUV (Sharma and Sharma, 1997).

1.2.1.2.2 Small unilamellar vesicles

SUV are defined as those consisting of single bilayer- liposomes presenting themselves at the lowest threshold of size possible for phospholipid vesicles. The size varies between 15nm and 100nm in size, depending on the ionic strength of the aqueous medium and the composition of lipid of membrane. For example, pure egg lecithin and DMPC in normal saline typically yields vesicles around 15nm and 25nm in size, respectively. The most common method of SUV preparation is via sonication of a suspension of MLV (Batzri and Korn, 1973; Kirby and Gregoriadis, 1999); an effective method of reducing the liposomal particle size for small batches due to its speed and simplicity (Liu *et al.*, 2001; Muller *et al.*, 2004) although disadvantages of this technique include the input of high energy hence inducing a risk of oxidation

(Muller *et al.*, 2004). Since, according to the above definition, they are at or close to the lower size limit, an advantage that SUV pose is a relatively homogenous population with respect to size (Liu *et al.*, 2001).

Due to their minute size, clearance of SUV from the systematic circulation is substantially decreased, therefore circulating for longer periods of time (Gregoriadis, 1985; Kirby and Gregoriadis, 1983) thus presenting an enhanced probability of exerting the desired therapeutic impact in tissues other than those in the RES (Kirby and Gregoriadis, 1999). However a disadvantage is their lower capacity for drug encapsulation owing to the small size (Kirby and Gregoriadis, 1999).



Figure 1.6 Size and number of bilayers dictating the type of liposome (Sharma and Sharma, 1997).

1.2.1.2.3 Large unilamellar vesicles

LUV are characterised by diameters of the order of magnitude in excess of 100nm and they comprise of a solitary bilayer (Liu *et al.*, 2001). As a consequence, for a given

concentration of lipid, LUV encapsulate a greater aqueous volume compared with SUV (Liu *et al.*, 2001). However, the absence of multiple lamellae renders the vesicle more fragile compared with MLV (Kirby and Gregoriadis, 1999).

1.2.1.3 Properties of liposomes affecting their role as carriers and therefore the solubilisation of hydrophobic drug

The structures and surface characteristics of liposomes encapsulating various drugs are influenced by lipid composition, lipid phase transition (membrane fluidity), adsorption of small molecules (i.e. ions) and large molecules (i.e. PEG), and amphiphilic drugs (Sharma and Sharma, 1997). Liposomes can be custom-designed for practically any requirement through varying of the lipid composition, size and surface charge (Ebrahim *et al.*, 2005). The influences of some of these parameters on liposomal performance are discussed below.

1.2.1.3.1 Membrane fluidity

Lipids possess a distinctive phase transition temperature (T_c), existing in different physical states below and beyond this threshold. Below the T_c , the lipids present themselves as a rigid and well-ordered 'solid' gel-like phase; whilst in contrast, the lipids exist in the liquid-crystalline 'fluid' phase above the transition temperature (Sharma and Sharma, 1997). A membrane that is rigid in nature is effective in reducing the bilayer efflux of drugs as well as stabilising the liposomes themselves (Massing and Fuxius, 2000). The degree of bilayer fluidity can be manipulated via the utilisation of lipids with various length of alkyl chain side group which correlates with transition temperature (Yoshioka *et al.*, 1994). Furthermore, the fluidity is also dependent on the nature (i.e. whether saturated or unsaturated) of the alkyl chain

moiety (Sharma and Sharma, 1997) or the presence of compounds such as cholesterol (Puglisi *et al.*, 1995). High levels of like cholesterol can totally eliminate phase transition inducing reduction of the membrane fluidity at a temperature greater than the T_c , which will yield more stable and less leaky vesicles (Massing and Fuxius, 2000; Sharma and Sharma, 1997). Investigations by Kirby and Gregoriadis (1981) found that after incorporating increasing amounts of cholesterol, leakage of solutes is decreased to a degree that even molecules as small as sucrose were retained within the vesicles.

The fluidity/rigidity balance is an important factor in determining bilayer behaviour *in vivo*. In general, the greater the rigidity of the membrane results in greater flexibility and fragility, while excessive levels fluidity could even result in membrane lysis (Hac-Wydro and Wydro, 2007). Kirby *et al.* (1980) demonstrated that plasma-induced destabilisation of cholesterol-free SUV was rapid and linked with practically total release of entrapped solutes. However, again the route of administration should be considered. For example, Oussoren *et al.* (1998) studied the effect of liposome stability on the protective influence of liposomes against dermal toxicity in mice following subcutaneous administration of doxorubicin entrapped in liposomes and free doxorubicin in formulations with relatively fluid bilayers (PC: cholesterol, 2:1 molar ratio) and liposomes with more rigid bilayers (DPPC: cholesterol, 2:1 molar ratio). Their experiments revealed that liposomes comprising of the more fluid lipid only prolonged the damaging effects of the drug whilst vesicles composed of a more rigid-state were significantly more effective in preventing local tissue damage over a longer period of time.

1.2.1.3.2 Size

The size and the number of bilayers of a liposome is a key characteristic which affects vesicular disposition (Drummond *et al.*, 1999) and the degree of solute encapsulation (Sharma and Sharma, 1997). The vesicle size is also an important parameter in determining the *in vivo* fate of vesicles (Kirby and Gregoriadis, 1999; Massing and Fuxius, 2000). It is generally accepted that for liposomes of similar composition, uptake by the RES is improved with increase in size (Drummond *et al.*, 1999). As discussed earlier (Section 1.2.1.2.2), as a result of their small size, *in vivo* clearance of SUV from the systematic circulation is substantially reduced, therefore circulating for longer periods of time thus presenting an enhanced probability of exerting the desired therapeutic impact in tissues other than those in the RES (Kirby and Gregoriadis, 1999). Uptake of liposomes by cells of the RES is induced by the binding (opsonisation) of serum proteins (e.g. complement factors or antibodies) to the lipids in the liposomal bilayer. Once opsonisation has materialised, liposomes can be swiftly recognised and become phagocytosed by the RES cells. The smaller liposomes are less vulnerable to the opsonisation by the serum proteins resulting in decreased recognition and thus a reduced clearing by the cells of the MPS (Massing and Fuxius, 2000).

1.2.1.3.3 Surface charge

Dispersion systems embody a vital group of pharmaceutical dosage forms for instance emulsions, suspensions, microspheres and liposomes. The dispersed phase can be either solid particles or immiscible droplets of liquid predominantly in mediums that are aqueous in nature. Through various mechanisms (i.e. via the ionisation of functional groups of the surface or the specific adsorption of ions) at the interface

between the dispersed phase and the aqueous medium, electrical charges are generated. These electrical charges play a key role in orchestrating the physical stability of the systems, particularly for those in the colloidal size range. For drug carrier systems such as liposomes, their *in vivo* fate and therapeutic efficacy are both influenced by these surface charges. Therefore, the understanding of this electrical occurrence becomes critical in the development of such a system (Li and Tian, 2006).

A charged solid particle in water containing electrolytes affects the distribution of ions in the solution. Oppositely charged ions to the charge on surface are attracted and firmly attached to the surface. This gives rise to an electric double layer consisting of the fixed charged surface and the diffuse layer that renders a net charge that is equivalent to that of the fixed layer but of opposite sign. The two regions are divided by a plane called the surface of shear (Figure 1.7). The quantitative measurement of the surface potential and the Stern potential are somewhat complicated. Thus, the potential between a static fluid plane surrounding the particle and the solution can be determined via the measurement of the mobility of the particle in an applied field. This potential is known as the zeta potential (ZP) (Heurtault *et al.*, 2003).

Values of the ZP of liposomes indirectly reflect vesicle surface net charge (Perrie and Gregoriadis, 2000) and can therefore reflect the nature of the lipid head-groups within liposomes and be used to evaluate the degree of interaction of the liposomal surface charges with the charges on entrapped/adsorbed drugs. Continued monitoring of vesicles will also allow the assessment of the physical stability of vesicular systems over time; the measurement of ZP is normally important in understanding dispersion and aggregation processes in applications (Heurtault *et al.*, 2003).

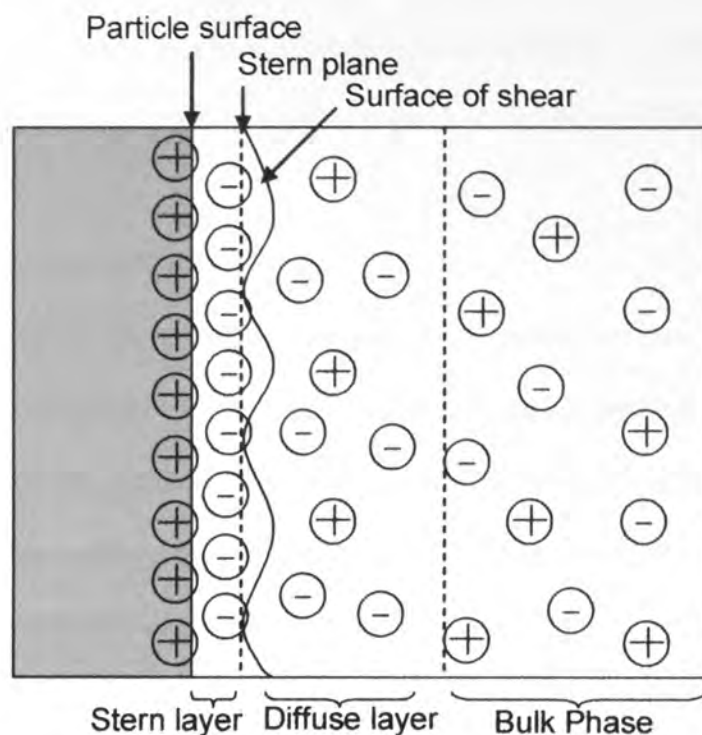


Figure 1.7 Schematic representation of the electric double layer occurring from charged particles in solution. Zeta potential is the potential between the surface of shear and the charged surface (modified from Kirby, 2007).

1.2.2 Niosomes

Niosomes are promising vehicle for drug delivery (Uchegbu and Vyas, 1998). Niosomes are analogous to liposomes and are those formed from non-ionic surfactants, which can deliver both lipophilic and hydrophilic drugs and behave like liposomes *in vivo*, prolonging the circulation of the entrapped drug. These species may offer an opportunity to use alternative materials to phospholipids since niosomes have been formulated from components such as Span 60, a permitted food additive, which is relatively inexpensive and broadly available. Thus, lower costs, enhanced stability and resultant ease of storage of non-ionic surfactants has resulted in the exploitation of niosomes as alternatives to phospholipids (Alsarra *et al.*, 2005). Furthermore, vesicle aggregation and fusion usually occur at a significantly lower rate for niosomes than for their liposomal counterparts (Carafa *et al.*, 1998). However,

formulation of niosomes is a time-consuming and inefficient process, particularly if small quantities are required for a particular application or dose (Biju *et al.*, 2006).

1.3 Method of preparation

Numerous methods have been constructed to satisfy a broad spectrum of requirements. These can be divided into two groups: those engaging in the creation of new bilayers via the removal of a lipid solubilising substance and those involving physical alteration of already generated bilayers (Kirby and Gregoriadis, 1999). An example for each kind is described below.

1.3.1 A method involving the generation of new bilayers: the thin-film technique

The standard thin film method first established by Bangham (Bangham *et al.*, 1965) is the most simple and widely utilised technique for MLV synthesis. The process involves the hydration of a thin film of lipids with an aqueous buffer at a temperature beyond the T_c of the main lipid used generating a heterogeneous population of vesicles. The drug intended for vesicle incorporation is included either in the aqueous hydration media (for hydrophilic agents) or amalgamated in the lipid film (for poorly water-soluble drugs) (Sharma and Sharma, 1997). A significant disadvantage of the thin-film method is its poor entrapment efficiency of hydrophilic drugs.

1.3.2 A method involving physical modification of already created bilayers: the dehydration-rehydration technique

Dehydration-rehydration vesicles (DRV) is a mild (i.e. organic solvents are not required) and simple method that can yield liposomes encapsulating high quantities of drug. A detailed method was proposed in 1984 as a simple technique for achieving a

high yield drug encapsulation in liposomes (Kirby and Gregoriadis, 1984). It was suggested as a procedure that is capable of incorporating a broad spectrum of substances using mild conditions, such as drugs, proteins and nucleic acids. The method is particularly suitable for large-scale industrial production (Gregoriadis, 1985; Kirby and Gregoriadis, 1984). The DRV method is based on the utilisation of dehydration and controlled rehydration thereafter, to bring about fusion of pre-generated liposomes (Kirby and Gregoriadis, 1984).

1.4 Investigation of liposomal systems using Langmuir monolayers

Langmuir monolayers have undergone scrutiny for more than a century (Kaganer *et al.*, 1999) as they have significant implications in many fields of science and technology such as in biology and material science (Dynarowicz- Latka *et al.*, 2001). They are useful for investigating intermolecular interactions between membrane components as well as membrane components and biomolecules such as drugs and proteins (Hac-Wydro and Wydro, 2007). Only amphiphilic molecules will form monolayers (Barnes and Gentle, 2005). They are constructed and characterised using the Langmuir trough, which consists of a trough, a flat container-like apparatus usually manufactured from hydrophilic substances like teflon to quarantine the subphase water and mobile barrier(s) that span across the surface water. An electronic device is used for measuring surface tension by the way of force exerted on a wetted Wilhemy plate submerged into the subphase. The Wilhemy method is rapid, uncomplicated and accurate with a standard experiment involving the placement of a known quantity of amphiphilic substance dissolved in a water-immiscible, volatile organic solvent (i.e. chloroform) on the surface of subphase water. Thereafter, the solvent is given sufficient time to evaporate and compression (or expansion) of the

resulting monolayer is induced via the mobile barrier(s) (Barnes and Gentle, 2005; Davis and Rideal, 1963; Dynarowicz-Latka *et al.*, 2001; Heurtault *et al.*, 2003).

The geometrical area of the surface region inhabited by monolayer and the amount of amphiphile on the surface yields the *area per molecule* (A) (Barnes and Gentle, 2005). The area per molecule is one of the key features of lipid membrane, playing a fundamental role in the ordering of alkyl chain moieties as well as the dynamics of lipids (Liu and Yang, 2006). The surface tension can be quantified either by utilising horizontal movable barrier(s) to sense the difference in tension between the monolayer-covered region on one side and the clean water surface on the corresponding side, or via measuring the vertical pull of surface tension on a Wilhemy plate (usually made of platinum or filter paper) partially immersed in the water (the absolute method) (Barnes and Gentle, 2005; Dynarowicz-Latka *et al.*, 2001). The outcome is usually reported as the surface pressure (Π ; the decreasing value of surface tension; equation 1.3) derived from:

$$\Pi = \gamma_w - \gamma_f \quad (1.3)$$

Where γ_w denotes the surface tension of clean water, and γ_f being the surface tension covered with monolayer (Barnes and Gentle, 2005). The standard Langmuir experiment yields an isotherm of surface pressure versus area per molecule. From the shape of such an isotherm, four principle monolayer phases can be identified: a gaseous phase, a liquid-expanded phase, with further compression generating a liquid-condensed phase, and the solid phase (Barnes and Gentle, 2005; Davies and Rideal, 1963; Minones Jr. *et al.*, 2006).

To attain reproducibility, rigorous conditions must be implemented during monolayer investigations (Dynarowicz-Latka *et al.*, 2001):

- a) pure substances, water and spreading solvents (preferably HPLC standard) must be used;
- b) application of appropriate (i.e. highly volatile and water immiscible) spreading solvents;
- c) accurate weighing of monolayer compounds;
- d) meticulous filtration of non-dissolved materials.

Langmuir monolayers are exceptional model systems for membrane biophysics, in view of the fact that a biological membrane can be assumed as two weakly coupled monolayers (Kaganer *et al.*, 1999), with two thermodynamical variables, temperature and pressure, being readily controlled (Castelli *et al.*, 2007). The range of surface pressures that are experimentally viable is limited by collapse of the monolayer. For example, during a constant-rate compression of monolayer, the process ends with the generation of a “monolayer collapse”; this is where material is forced out of the surface resulting in the appearance of three-dimensional structures (Barnes and Gentle, 2005; Gaines, 1966; Kaganer *et al.*, 1999). The collapse pressure is the highest pressure to which a monolayer can undergo compression before molecules are expelled to form a new phase (Gaines, 1966). The collapse pressure is therefore an excellent tool in assessing monolayer stability as the monolayer stability limit may be defined as the maximum pressure achievable in the film without the possible event of collapse (Gaines, 1966). The extrapolated molecular area (\bar{A}) is determined by the way of extrapolating the slope of the monolayer π - A isotherm at high surface pressure

to zero pressure. The hypothetical space inhabited by a single molecule in the condensed phase is given by the point at which this slope intersects the x-axis (Zhao and Feng, 2006).

1.5 The model drug candidates

For the development of liposomes as possible carriers for low solubility drug a selection of model drug candidates from two different groups of compounds were investigated.

1.5.1 Basic drugs

Bronsted-Lowry defines a base as an acceptor of protons (Cairns, 2003). Under aqueous conditions, drugs will perform as bases only if they consist of a “nitrogen atom with a lone pair of electrons available for reaction with protons” (Cairns, 2003). At a low pH, a basic drug, such as ranitidine, is predominantly in the ionic form, which is normally very soluble in aqueous media. As the pH is elevated, more undissociated base is generated, which when exceeds the limited water solubility of this form, precipitation of free base occurs from the solution (Martin *et al.*, 1993).

1.5.1.1 Diazepam

Diazepam (Figure 1.8) is a highly lipophilic drug (Log P = 2.99; Wishart *et al.*, 2006) with a molecular weight (MW) of 285Da. The drug belongs to a class of neuroactive compounds that enjoy a wide administration due to their hypnotic, muscle relaxant, anticonvulsant and anxiolytic features (Omran *et al.*, 2001).

Studies carried out by Fatouros and Antimisiaris (2002) revealed that diazepam-incorporating liposomes show a substantial effect on the vesicle zeta-potential and

stability. The liposomes had a negative surface charge, while their membrane integrity was substantially higher when compared with that of empty vesicles.

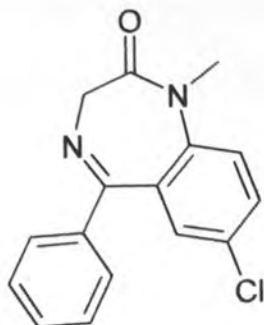


Figure 1.8 Structure of diazepam.

1.5.1.2 Midazolam

Midazolam (MW = 326Da; Figure 1.9) is an agent commonly utilised in the emergency sector to provide safe and effective sedation prior to procedures such as reduction of dislocations and laceration repair. It has a Log P value of 3.87 (Wishart *et al.*, 2006). The drug is also useful in the treatment of status epilepticus, generalised seizures, and behavioural emergencies, especially in cases where intravenous access is not available (Nordt *et al.*, 1997). The nitrogen in the imidazole ring renders the structure basic (Andersin and Tammilehto, 1995).

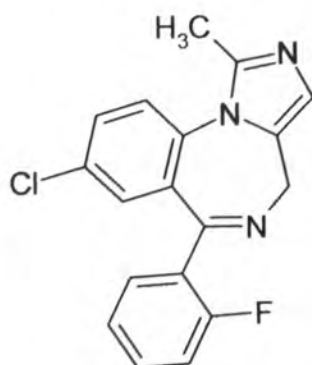


Figure 1.9 Structure of midazolam.

1.5.2 Acidic drugs

A Bronsted-Lowry definition of an acid is a substance that undergoes ionisation that results in the donation of protons to its surroundings (Cairns, 2003). Drugs that are

rendered acidic, such as the non-steroidal anti-inflammatory agents, are soluble to a lesser extent in acidic solutions as opposed to alkaline solutions since the principal undissociated species cannot interact with water molecules to the same degree as the ionised form, which readily undergoes hydration (Florence and Attwood, 1988).

1.5.2.1 Ibuprofen

Ibuprofen (MW = 206Da; Figure 1.10) is a propionic acid derivative that was the inaugural non-steroidal anti-inflammatory drug to be commonly prescribed for therapy of pain and inflammation (Farrar *et al.*, 2002). The drug is characterised by a Log P value of 3.48 (Wishart *et al.*, 2006). Ibuprofen suffers from inherent formulation problems, such as poor compaction low solubility (Whelan *et al.*, 2002).

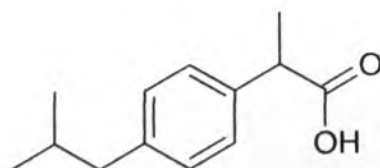


Figure 1.10 Structure of ibuprofen.

1.5.2.2 Phenytoin

Phenytoin (Figure 1.11) is an effective anticonvulsant agent, which is one of the most comprehensively used in epilepsy therapy. The drug is a weak acid with a MW of 252Da and is practically insoluble in water (theoretical Log P = 2.24; Wishart *et al.*, 2006). Despite the existence of significant experimental evidence for the effectiveness of phenytoin in the treatment of number convulsive disorders, problems remain to be addressed with respect to absorption, pharmacokinetic, and safety difficulties.

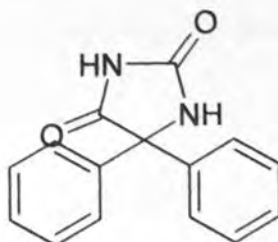


Figure 1.11 Structure of phenytoin.

As a result of its poor solubility in water and insufficient dissolution rate, phenytoin displays unsatisfactory and erratic bioavailability after oral administration to patients. For parenteral utilisation, the drug has been formulated as a salt of sodium in an aqueous alkaline medium (at pH \sim 12) consisting of 40% propylene and 10% ethanol. However, there is the existence of a serious danger of phenytoin precipitation at the site of injection because, in physiological media (pH $<$ 8), the sodium salt yields the corresponding insoluble free acid.

The therapeutic potential of phenytoin could be enhanced via the examination of methods employed for improving solubility and/or dissolution rate of poor soluble compounds (Latrofa *et al.*, 2001; Scriba *et al.*, 1997).

1.5.2.3 Propofol

Propofol (MW = 178; Figure 1.12), 2,6-diisopropylphenol, is an intravenous drug utilised for anaesthesia (Altomare *et al.*, 2003). Due to the drug's hydrophobic character (Log P = 3.84; Wishart *et al.*, 2006), propofol was initially formulated as a 1% (w/v) solution in the presence of the solubilising surfactant cremophor el. However complications such as anaphylactic reactions have led to the search for improved formulations. At present, formulation of propofol is as an oil-in-water

emulsion (1%, o/w) of soya bean oil, glycerol and purified egg phosphatide (Diprivan®, Zeneca, UK).

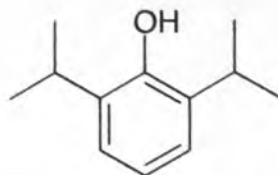


Figure 1.12 Structure of propofol.

As a lipid-based emulsion, Diprivan® suffers from various drawbacks, such as poor physical stability, potential for embolism, and the requirement for aseptic handling (Altomare *et al.*, 2003). Therefore, formulations consisting of PC derivative and cholesterol could be investigated as an alternative delivery vehicle. Muller *et al.* (2004) have successfully synthesised liposomes encapsulating local anaesthetics, from soybean lecithin Phospholipin® 90 G. Momo *et al.* (2002) demonstrate propofol to be highly lipophilic and absorbs almost entirely in the lipid phase of dimyristoylphosphatidylcholine and egg yolk PC multilamellar liposomes.

1.5.2.4 Rifampicin

Rifampicin (Figure 1.13) is the main component of TB chemotherapy in association with other front line anti-TB drugs and is utilised in both intensive and continuation phase for the therapy of all categories of patients, but requires a high-dose drug therapy spanning a period of 4-6 months (Vyas *et al.*, 2004). Rifampicin is an acidic drug (Ovcharova *et al.*, 1990) which has a MW of 823Da and a Log P value of 3.72 (Wishart *et al.*, 2006).

Rifampicin molecule possesses two functional groups of interest, the phenolic hydroxyl groups ($pK_a = 1.7$), and the nitrogen atoms of piperazine ($pK_a = 6.8$)

(Pasquardini *et al.*, 2008). The drug has several side-effects at dose levels administered in long-term clinical treatments, including immunological disturbances, rheumatoid, allergic rashes, eosinophilia, leukopenia, and other hepatotoxic complications (Vyas *et al.*, 2004).

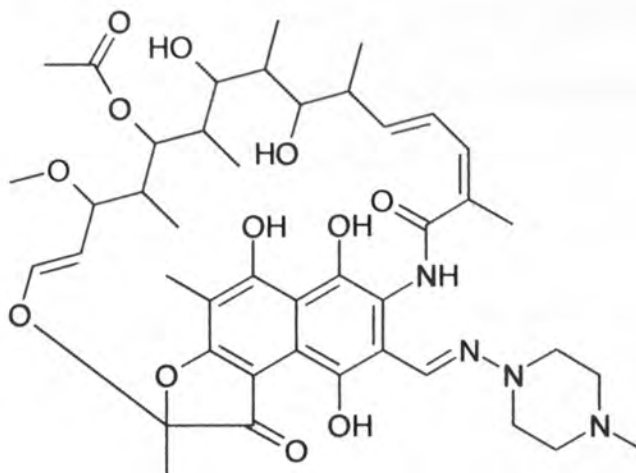


Figure 1.13 Structure of rifampicin.

1.6 Lipids used to investigate the solubilisation potential of liposomes

Amphiphilic lipid molecules, such as non-ionic surfactants and phospholipids, are made of a lipophilic part connected to a hydrophilic moiety. These compounds contain physicochemical properties of self-aggregation, solubilisation or emulsification (Ascarateil and Dupuis, 2006).

1.6.1 Non-ionic surfactants – alkyl esters

Surfactants are surface active agents whose chemical structures are typified by having two areas, one hydrophobic (hydrocarbon chain) and the other hydrophilic (an ionisable, polar or water soluble moiety) (Di Profio *et al.*, 2005). As they are attracted to both aqueous and oil phases, they are frequently labelled as being amphiphilic (Buckton, 1995). The principal purpose of surfactants is to act as wetting agents, i.e.

promote interfacial contact between a liquid and another phase. Surfactants lower the surface tension of a liquid via adsorption of these molecules at the surface of the liquid, and the adsorption to liquid-liquid interface resulting in the creation of emulsions. An additional characteristic of surfactant molecules is that they tend to form various kinds of supramolecular structures (Di Profio, 2005) and in excessive concentrations in solution they will favour association into micelles (Barnes and Gentle, 2005). There are four groups of surfactants:

- 1) Anionics- their hydrophilic segment of structure harbours a negative charge (e.g. sodium lauryl sulphate);
- 2) Cationics- the surface active component is the cation (e.g. benzalkonium chloride);
- 3) Ampholytics- surfactants that can act either as cationic or anionic structures;
- 4) Non-ionics- surfactants which consist of a hydrophilic section which is not of ionic nature (e.g. cetomacrogol) (Barnes and Gentle, 2005).

If non-ionic molecules are supplemented with cholesterol (a stabiliser), it is possible for stable bilayer structures to form that are known as niosomes (Buckton, 1995). Niosomes, originally devised by L'Oreal (Vanlerberghe *et al.*, 1972), are synthesised from the self-assembly of non-ionic amphiphiles in aqueous solution, usually involving some introduction of energy such as of a thermal nature or physical agitation (Uchegbu and Vyas, 1998). Alkyl esters, such as the sorbitan esters, are non-ionic surfactants extensively used in foodstuffs, and have previously been studied as the basis of non-ionic surfactant vesicles (Manosroi *et al.*, 2003; Yoshioka *et al.*,

1994). Sorbitan esters are made of sorbitol and an oleic acid linked by ester bonds and ethoxylated ones (polysorbates); they can be sorbitan mono or tri-oleate (Ascarateil and Dupuis, 2006).

1.6.2 Phospholipids

Phospholipids are essential lipid components of biological membranes that contain phosphoric residues (Wang *et al.*, 2003), where they play a main role in regulating cell function structural processes as well as in the recognition of molecules (Minones Jr. *et al.*, 2006). The presence of phosphorus in lipids extracted with ethanol from brain tissue was originally discovered in the early 1811. Substances of a similar nature were isolated from brain tissues with hot alcohol were named *matière blanche*, *cérébrote*, *acide cérébrique* or oleophosphoric acid. Later, in 1874, a phosphorous-containing lipid was isolated from egg-yolk and brain tissues, and was named phosphatidylcholine (PC) (Suzumura, 2005). PC and its derivatives (Figure 1.14) are amphiphilic molecules possessing polar head groups and non-polar lipid chains, self-associating in aqueous solutions at concentrations above their CMC (Wang *et al.*, 2003). Their amphiphilic feature and high surface activity allow phospholipids to be used in the food industry to assemble bilayer lipid-protein membranes to stabilise emulsions (Li *et al.*, 1998).

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), are synthetic phospholipids derived from PC. Each contains two hydrophobic alkyl chains (14-carbon (C), 16-carbon and 18-carbon, respectively) attached to a phosphate moiety, which is hydrophilic in nature. The phospholipids 1-palmitoyl-2-oleoyl-sn-

glycero-3-phosphocholine (POPC) and 1-myristoyl-2-stearoyl-sn-glycero-3-phosphocholine (MSPC) are also derivatives of PC but unlike the above mentioned, they are structurally asymmetrical. POPC is a PC derivative that possesses a C_{16} hydrocarbon chain (as in DPPC) paralleling a C_{18} (as in DSPC) one. Whilst, MSPC also carries C_{18} alkyl chain group; but unlike in the case for POPC, this particular hydrocarbon series also possesses a sole unsaturated bond that is paired with a C_{14} alkyl side-chain (as akin to DMPC).

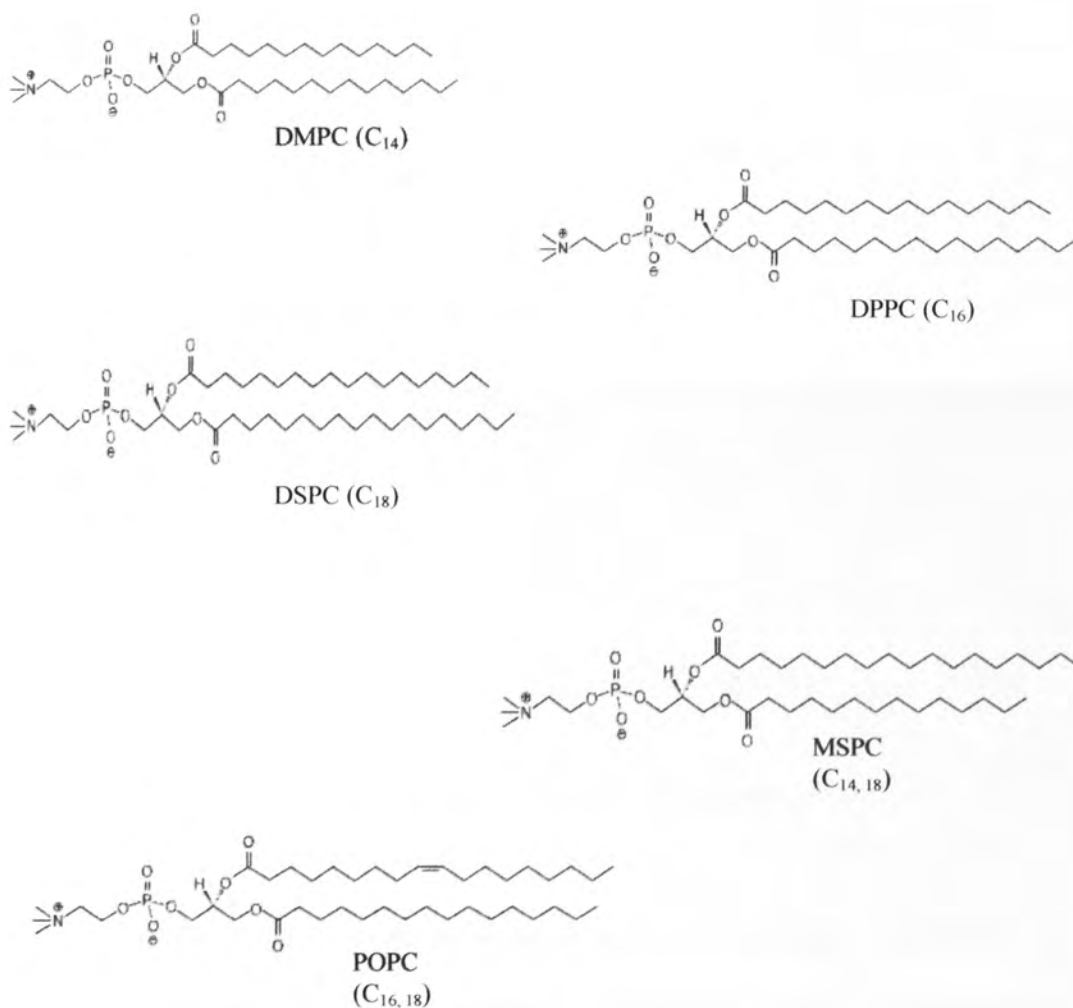


Figure 1.14 Structures of various analogues of PC.

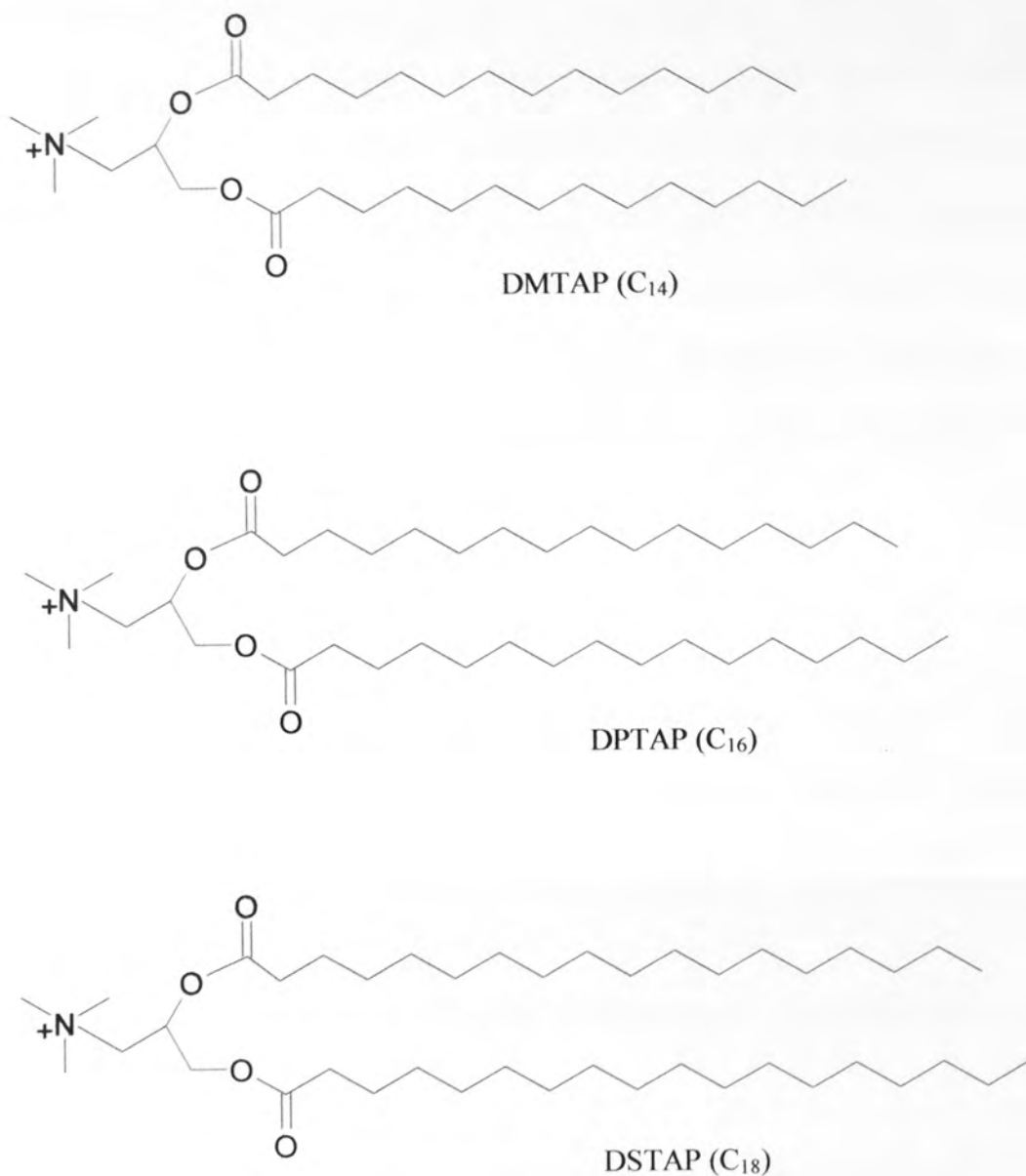


Figure 1.15 Structures of various derivatives of TAP.

Cationic lipids (1,2-dimyristoyl-3-trimethylammonium-propane, DMTAP; 1,2-dipalmitoyl-3-trimethylammonium-propane, DPTAP; and 1,2-distearoyl-3-trimethylammonium-propane, DSTAP; Figure 1.15) were also utilised in the synthesis of MLV. The TAP lipids possess a head-group containing a positively charged trimethylammonium moiety that renders the lipid an overall cationic charge.

1.7 Aims and objectives

A lot of research has been carried out with liposomes as carriers for hydrophilic drugs (e.g. Carafa *et al.*, 1998 and Yoshioka *et al.*, 1994); however limited attention has been given to poorly water-soluble drugs. To be effective as a carrier a systematic investigation of the parameters that control the physico-chemical characteristics of liposomes containing bilayer-loaded drugs is required. Therefore the overall aim of this research was to understand the mechanistic processes involved in the construction of liposomes that can be used as solubilisation agents.

To achieve this, the objectives were:

- To use a series of model poorly soluble drugs to determine the physicochemical characteristics and compositions of surfactant-based colloidal systems which promote hydrophobic drug loading and retention within the bilayer under various conditions;
- To correlate this with the molecular characteristics of the incorporated drug and the colloid composition;
- To investigate and model the interaction of the drug molecule with the surfactant bilayer;
- To understand and use these relationships so as to better design and engineer solubility enhancing colloidal systems tailored to the need of the drug and the route of administration.

Chapter Two:

Materials and methods

2.0 Materials and methods

2.1 Materials

2.1.1 Chemicals

Propofol was supplied by MP Biomedicals, LLC, Eschwege, Germany. Diazepam, ibuprofen, indomethacin, midazolam, phenytoin rifampicin, cholesterol (Chol; >99%), dicetylphosphate (DCP), EDTA, Trypan Blue, phosphate buffer saline (PBS) tablets, deuterated water (D₂O), Sephadex® G-50, uranyl acetate, dimethyl sulfoxide (DMSO), Tritox-X, tetradecanol (Tet) and octadecanol (Oct) were purchased from Sigma-Aldrich, Dorset, UK.

Hexadecanol (Hex) was supplied by Fluka Chemie, Bucks SG. England. Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin/L-glutamine were supplied by Invitrogen Corporation, Paisley, Scotland. Radioactive indomethacin was purchased from Perkin Elmer Life Sciences, Boston, Massachusetts, USA. Fresh defibrinated sheep red blood cells (RBC) were from TCS Biosciences Ltd, Botolph Claydon Buckingham, UK. The MTS reagent was supplied by Promega, Madison, Wisconsin, USA. All the chemicals utilised were of analytical grade.

2.1.2 Lipids and surfactants

Egg phosphatidylcholine (PC; grade I), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Lipid Products, Epsom, Surrey, UK. Dilignoceroyl phosphatidylcholine (C₂₄PC), 1,2-distearoyl-3-phosphatidylglycerol (DSPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

(POPC), 1-myristoyl-2-stearoyl-sn-glycero-3-phosphocholine (MSPC), 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) and 1,2-distearoyl-3-trimethylammonium-propane (DSTAP) were supplied by Avanti Polar Lipids, Inc., Alabaster, Alabama, USA. The partially/fully deuterated lipids 1,2-dimyristoyl-D54-sn-glycerol-3-phosphocholine-1,1,2,2-D4-N,N,N-trimethyl-D9 (DMPC [PCD₆₇]), 1,2-dimyristoyl-D54-*sn*-glycero-3-phosphocholine (DMPC [PCD₅₄]), 1,2-distearoyl-D70-*sn*-glycero-3-phosphocholine-1,1,2,2-D4-N,N,N-trimethyl-D9 (DSPC [PCD₈₃]) and 1,2-distearoyl-D70-*sn*-glycero-3-phosphocholine (DSPC [PCD₇₀]) were also supplied by Avanti Polar Lipids, Inc., Alabaster, Alabama, USA.

Sorbitan monolaurate (Span 20) and sorbitan trioleate (Span 85) were purchased from Sigma-Aldrich, Dorset, UK. Sorbitan monopalmitate (Span 40) and Sorbitan monooleate (Span 80) were supplied by Fluka Chemie, Bucks SG. Sorbitan monostearate (Span 60) was a product of Lancaster, Eastgate, Morecambe, England.

2.2 Methods

2.2.1 Preparation of multilamellar vesicles (MLV)

MLV were generated using a technique based on the standard film method (Bangham *et al.*, 1965). Briefly the lipid entities were dissolved in a chloroform: methanol (9:1) solvent mixture with and without the required amount of drug (1.00mg) (Figure 2.1a). A mixture of chloroform and methanol was used to improve solubility of the components. Thereafter, the solvents were evaporated on a rotary evaporator (Buchi rotavapor-R) to yield a dry film (Figure 2.1b). The film was flushed with N₂ to ensure complete solvent evaporation and subsequently hydrated above the liquid-crystalline

temperature (T_c) of the lipids (with frequent vortexing and intervals of rest) with 2mL of double distilled water to give final lipid concentration of 16-24 μ mol/mL dependent on formulation (Figure 2.1c). Thereafter, the preparations were left to stand above the T_c for 30min during which time the vesicles were formed (Figure 2.1d).

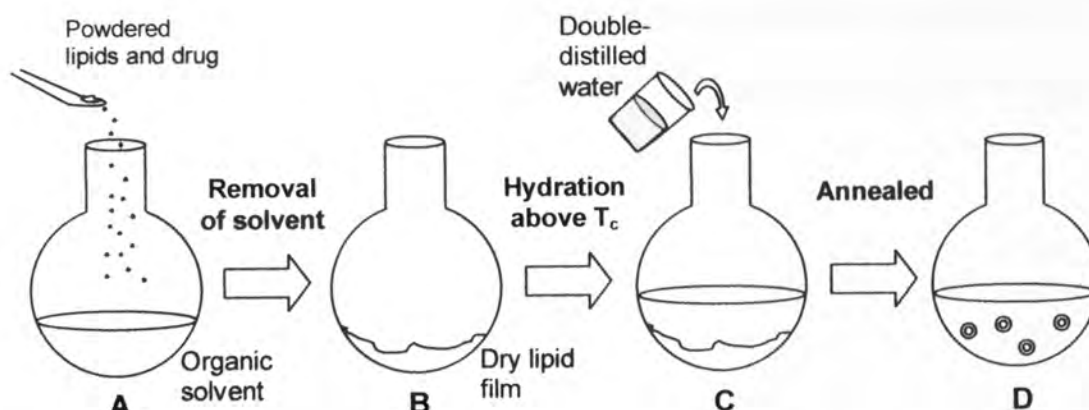


Figure 2.1 Schematic representation of the established 'hand-shaking' method. Lipids and drugs were dissolved in solvents (a) and then evaporated (b); thereafter, dried lipid film was hydrated in double-distilled water (c) and finally left to stand for 30min to yield vesicles (d). (Diagram modified from Kirby, 2007)

2.2.2 Preparation of small unilamellar vesicles (SUV)

The MLV suspension (prepared as detailed in Section 2.2.1) was sonicated using a titanium probe (exponential micro-probe and diameter 3mm) slightly immersed into the suspension for 2min at amplitude of 5microns using a MSE probe sonicator to yield a clear suspension of small unilamellar vesicles ranging in the size of 75-100nm in diameter. After sonication, the preparations were left to stand above the T_c for 30min.

2.2.3 Determination of drug encapsulation efficiency in MLV

The drug loading of MLV was determined by measuring the non-incorporated drug present in 30mL wash media (60mL in case of diazepam; PBS, 0.01M) following

separation of liposomes by centrifugation (Beckman J2 Centrifuge) at 27,200g for 30min (Mohammed *et al.*, 2007). This washing stage was repeated twice to ensure maximum possible separation of free drug from liposomes (Casals *et al.*, 1996). The supernatant containing the unincorporated drug was analysed by UV spectroscopy (Unicam Helios) at 212-268nm (Table 2.1) wavelength following confirmation by wave-scan for optimum absorption peaks and respective calibration. All samples were diluted appropriately in phosphate buffered saline (with respect to solubility values) to avoid drug precipitation.

Drug	Wavelength studied (nm)	R ²
Diazepam	228	0.9973
Ibuprofen	221	0.9962
Indomethacin	316	0.9971
Midazoalam	258	0.9901
Phenytoin	212	0.9987
Propofol	268	0.9965
Rifampicin	236	0.9970

Table 2.1 The wavelengths for the drugs studied as determined via wave-scan using a Unicam Helios.

From the amount of drug and lipid contained in the vesicles, the encapsulation efficiency was determined as the percentage of initial drug (equation 2.1) and the relationship of percentage mol of drug to mol of lipid present (equation 2.2). However, entrapment was predominantly presented as per the latter equation. This parameter was used to compare the ability of different compositions to encapsulate hydrophobic model drug candidates. The encapsulation efficiency values expressed as entrapment percentage for the prepared MLV was calculated through the following relationships:

Equation 2.1: As percentage of initial drug (according to Bhatia, 2004, Hathout *et al.*, 2007 and Terzano *et al.*, 2005):

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Mass of entrapped drug}}{\text{Mass used for MLV preparation}} \times 100$$

Equation 2.2: As the molar amount of drug per mole of lipid (% mol/mol) (Edwards and Baeumner, 2006):

$$\text{Encapsulation efficiency (\% mol/mol)} = \frac{\text{Mole of entrapped drug}}{\text{Total moles of lipid}} \times 100$$

2.2.4 Determination of encapsulation efficiency using an alternative UV-based method

The protocol in Section 2.2.3 was validated by a direct method previously established by Fatouros and Antimisiaris (2002) analysing propofol concentration within a selected set of liposome preparations. The MLV pellet obtained as detailed in Section 2.2.1 was lysed with isopropanol in a 50/50 v/v mixture and solubilised by vortexing. The analysis was performed using UV. For this, standard calibrators of drug were prepared in the appropriate concentration range (in same media) and spiked with drug-free MLV as to attain the same final lipid concentration as in the samples.

2.2.5 Determination of encapsulation efficiency using HPLC

The method detailed in Section 2.2.3 was further validated using high performance liquid chromatography (HPLC; Dionex GP50-2, Dionex Softron GmbH, Germering, Germany) with UV detection using an adapted method of Terzano *et al.* (2005) after separating the drug-loaded pellet from the supernatant as described in Section 2.2.1. Thereafter, 1mL MLV solution was spiked with 1mL isopropanol, theoretically disintegrating the entire lipid bilayer (therefore releasing all entrapped drug), and

assayed for drug content. Drug content was measured using HPLC at 221nm or 258nm (ibuprofen or midazolam, respectively, as determined via wave-scan) (using methanol: double distilled water; 60:40 as the mobile phase). The flow rate was 1.0mL/min and the injection volume was 15 μ L. The standard calibration curves were prepared in PBS with known amounts of drug and processed as described above with R^2 values 0.99 for both curves.

2.2.6 Determination of entrapment efficiency of indomethacin using radio-labelled [14 C] indomethacin

The protocol in Section 2.2.3 was additionally validated by a direct method using a radioactive isotope of carbon-14 (14 C). MLV incorporating indomethacin was prepared as described in Section 2.2.1. MLV was spiked with radio-labelled [14 C] indomethacin; the free drug was separated from the encapsulated drug by passing the MLV through a column pre-packed with Sephadex (G50) (which had previously been equilibrated with PBS). Encapsulation efficiency was determined (using a beta scintillation counter) by collecting the fractions of 1.0mL aliquots through a Sephadex G-50 column and then calculating as the percentage of the total radioactivity associated with the liposome fraction.

2.2.7 Preparation of standard curve

Each standard drug was accurately weighted, then dissolved in methanol and diluted with PBS (pH = 7.4) to appropriate concentration. A stock-solution of 100ug/mL in methanol was prepared and from this a range of standard solutions (calibrators) were made for all drugs in PBS. The maximum concentration of calibrator for each drug was selected so that an absorbance of around 1.0-1.2 was achieved, which generally is

accepted to be the threshold beyond which Beer-Lambert linearity is lost. The linearity of a method is a measure of the range within which the observed detector response (y) are directly proportional to the analyte concentration (x) in samples within a given range (expressed as a linear regression equation: $y = a + bx$) (Cao *et al.*, 2005). As a consequence, the range of standard solutions varied with each drug- 1.0–15.0 $\mu\text{g/mL}$ for diazepam, 5.0–25.0 $\mu\text{g/mL}$ for ibuprofen, 5.0–30.0 $\mu\text{g/mL}$ for indomethacin, 5.0–50.0 $\mu\text{g/mL}$ for midazolam, 5.0–30.0 $\mu\text{g/mL}$ for phenytoin, 5.0–100.0 $\mu\text{g/mL}$ for propofol and 5.0–30.0 $\mu\text{g/mL}$ for rifampicin.

The optimum wavelength of absorbance for each drug (Table 2.1) was determined via wave-scans of each drug couple with literature back-up. Results of the regression analyses and the correlation coefficients (R^2) are listed in Table 2.1. The high correlation coefficient values for all drugs ($R^2 > 0.99$) indicated good linearity between their detector absorbance and drug concentrations. To control inter-day variation, standards were run each day of investigation.

2.2.8 Determination of vesicle volume distribution

The vesicle mean volume distribution (D[4,3]) of liposomes and niosomes were determined by light scattering based on laser diffraction spectroscopy using a Malvern Mastersizer by diluting the sample appropriately with double-distilled water. The apparatus consists of a small volume-holding cell with a magnetic bead constantly stirring to maintain the sample in suspension. In later studies, a sympatec Helos/BF laser diffraction was used to determine mean volume distribution of MLV suspensions as stated above.

The z-average diameter of SUV were determined using a Zetaplus (Brookhaven Instruments, UK) with 100 μ L of the suspension diluted to 4ml using double-distilled water and recorded at 25°C.

2.2.9 Determination of vesicle zeta potential

Zeta potential, an indirect measurement of the surface charge of vesicle (Perrie *et al.*, 2004), was measured by photon correlation spectroscopy using the same Zetaplus in 0.001M PBS (pH = 7.4) at 25°C. The dispersion was diluted accordingly in PBS and 4mL of this was commenced for analysis. The reported measurements were the average figures of ten readings.

2.2.10 Drug release studies

The release rate of drug was determined by incubating drug-loaded vesicles (after separation of the free drug) in 30mL PBS at 37°C in a shaking (constant) water bath. Initially, three independent samples each containing 56 μ mol liposomes were prepared. Each homogenous suspension was divided into seven batches each containing 8 μ mol of preparation. At time intervals of 0, 2, 4, 8, 24, 48, 72hour, the medium was centrifuged at 27,200g for 30min. The supernatant was analysed spectrophotometrically at appropriate wavelength and the amount of drug released was assayed by comparison with a calibration curve for drug in PBS.

2.2.11 Liposome stability studies

Liposomal size and drug retention were used as parameters to preliminarily indicate the physical stability of liposomes (protocol adapted from du Plessis *et al.*, 1996 and Vangala *et al.*, 2006). The stability of formulations, with respect to retention of the

entrapped drug and changes in the size distribution, was determined by vesicles (after separation of the free drug) in 10mL PBS at 4°C and 25°C. Initially, three independent samples of 40mL MLV containing 36µmol liposomes were prepared. Each homogenous suspension was divided into four batches each containing 10mL (9µmol) of preparation. At time intervals of 0 (immediately after preparation), 7, 14 and 28day the vesicle size was determined as outlined in Section 2.2.8, with retention measured as described in Section 2.2.3

2.2.12 Statistical analysis

The treatment of the experimental results was based on the analysis average and the analysis of variance (ANOVA). Thereafter, Bonferroni's multiple *t*-test and Student-Newman-Keuls Test were used to compare the difference between the various encapsulation efficiencies, MLV size and zeta potential of the formulations (Mohammed *et al.*, 2007). Differences were considered significant when $P < 0.05$. Each value was obtained from at least triplicate samples and expressed as mean \pm S.D.

2.2.13 Light microscopy analysis

Light microscopy of liposomes (prepared as described in Section 2.2.1) were examined at room temperature under a visible microscope (Axioskop 40) with a 40x objective lens fitted with an Axio high resolution camera at various magnifications, so as to substantiate the formation and to understand the nature of the vesicles. Images were captured using Axiovision version 3.1 software (Gopinath *et al.*, 2004).

2.2.14 Transmission electron microscopy (TEM) analysis

Liposomes were morphologically examined by TEM using JEOL 1200EX transmission electron microscope fitted with a LaB6 filament, with an operating voltage of 40-120kV. A small drop of liposome formulation (10 μ L) was placed on a copper grid covered in a polymer film and allowed to stand for 2min. Excess sample was removed using filter paper, followed by addition of 10 μ L of uranyl acetate. The grid was subsequently allowed to stand for a further 2min, washed in distilled water and air dried, resulting in the formation of a thin film of stained vesicles, which was then viewed at an operating voltage of 70kV.

2.2.15 Environmental electron- scanning microscopy (ESEM) analysis

Morphological characteristics and stability of liposomes were carried out using a Philips Electron Optics ESEM. The ESEM sample container was loaded with liposome formulation previously hydrated in double-distilled water and examined under saturated water vapour conditions. Dynamic stability of MLV was monitored by gradual decrease of pressure in the sample chamber resulting in a controlled dehydration of the sample environment. A working temperature of 5°C was maintained throughout.

The effect of drug loading on liposome stability was analysed by gradual increase of temperature in the sample chamber yielding a controlled dehydration of the lipid films, whilst maintaining a constant operating pressure of 5.0Torr.

2.2.16 Langmuir studies

An automated controlled film balance apparatus (KSV Langmuir Mini-trough, KSV Instruments Ltd., Helsinki, Finland) equipped with a platinum Wilhemy plate and

placed on a vibration-free table was used to collect the surface pressure-area isotherms. The size of the trough was 24225.0mm² enclosing a total volume of about 220mL; the subphase was filtered double-distilled water. The investigated substances were kept in tightly closed bottles in the freezer. Mixed solutions were prepared by thoroughly mixing appropriate quantities of respective stock samples. The compounds (at fixed total concentration of 1mg/mL) were dissolved in chloroform and 20μL of each solution was spread onto the air/water interface with a Hamilton microsyringe, precise to $\pm 0.2\mu\text{L}$. After spreading, the monolayers were left for 10min for the chloroform to evaporate. Thereafter, the molecules underwent constant compression until the required surface pressure was attained. Optimisation work revealed that a routine compression speed of 10mm/min yielded the best isotherms therefore this speed was maintained throughout all monolayer studies. The temperature of the subphase was kept constant to $20 \pm 1^\circ\text{C}$ by means of an external water bath circulation system. Each experiment was only compressed once and performed at least three times with monolayers prepared from different solutions. KSV software (KSV Instruments Ltd, Helsinki, Finland) was used for data analysis.

2.2.17 MTT assay

2.2.17.1 Resuscitation of frozen COS-7 cell line

An ampoule comprising COS-7 cells (African green monkey kidney fibroblast cells) was removed from liquid nitrogen storage and semi-immersed in a 37°C water bath for approximately 2min, until cells have thawed. The exterior of the ampoule was wiped with tissue paper (moistened with 70% isopropanol) prior to being placed within a sterile microbiological safety cabinet in order to maintain sterile conditions. Under sterile conditions, the ampoule lid was removed and cell solution removed and

gradually pipetted into a 75cm² cell culture flask containing 20mL Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin/L-Glutamine, in order to dilute the toxic effects of dimethyl sulfoxide (DMSO). Cells were incubated at 37°C under sterile conditions and 5% CO₂ atmosphere.

2.2.17.2 Subculture of COS-7 cells

The DMEM was removed by meticulous suction, followed by the addition of 5mL trypsin/EDTA, ensuring that all cells are covered, which then underwent incubation at 37°C for 5min. An inverted microscope was used to examine the cells to ensure all cells have detached from the flask. Thereafter, 5mL of DMEM was added to the cell suspension to dilute the trypsin/EDTA solution and the cell suspension was transferred to a 50mL centrifuge tube followed by centrifugation (Mistral 3000i) at 200g for 10min at a temperature of 15°C. Following centrifugation, the supernatant was separated under sterile conditions, with the resultant pellet resuspended in 10mL DMEM. 1mL of the cell suspension was transferred to a 75cm² cell culture flask containing 19mL DMEM and incubated under sterile conditions, in a humid 5% CO₂ conditions.

2.2.17.3 Cell quantification

From the cell suspension of COS-7 cells, 200µL was removed and to this 200µL trypan blue was added and mixed. Using a haemocytometer, 10µL of the trypan blue cell suspension was added and viewed microscopically. The bright cells (i.e. viable cells) were counted and from this the cells concentration was calculated (including dilutions), whereas the cells stained blue indicate non-viable cells.

2.2.17.4 Cell preparation and plating for in vitro studies

Adherent COS-7 cells were brought into suspension and quantified as detailed in Section 2.2.17.3. The cell suspension was then centrifuged at 200 x g for 10min at 15°C and the pellet was re-suspended to a cell concentration of 1×10^6 cells/mL with supplemented DMEM. For cytotoxicity assay, 100µL of cell suspension was pipetted per well on a 96-well plate. Culture plates were then incubated at 37°C for 24hour.

2.2.17.5 Samples preparation

COS-7 cells, plated 24hour previously, were removed from the 37°C incubator and the media was removed, subsequently washed with 100µL pre-warmed opti-MEM. 100µL of liposome suspension (prepared as described in Section 2.2.1) was added to the cells and incubated at 37°C for 5hour in a humid CO₂ atmosphere.

2.2.17.6 Cytotoxicity assay

To each well of a 96-well plate containing COS-7 cells, 20µL of MTT reagent was added. The MTT reagent is bio-reduced by the cells into a formazan product, which indicates the presence of metabolically active cells. Plates were then gently mixed and incubated for 4hour at 37°C, in a 5% humid CO₂ atmosphere. After 4hour incubation, the quantity of formazan produced was measured on microplate reader (Bio-Rad, model 680) at A₄₉₀, with the absorbance reading being directly proportional to the number of living cells in the medium. Results are compared to and expressed as percentage of negative control (i.e. cells and medium). Positive control was also included which was cells incubated Triton X-100.

2.2.18 Red blood cell (RBC) assay

Membrane-disruptive action of liposomes was tested by haemolysis assay (Chen *et al.*, 2005; Kheirloomoom *et al.*, 2005). Liposomes were prepared in isotonic buffer (PBS; pH = 7.4) at a concentration of 18 μ moles/mL. Fresh defibrinated sheep red blood cells (RBC) were obtained. Erythrocytes were separated from platelet rich plasma by centrifugation (330g, 14min). The upper plasma and white buffy coats were removed by a syringe and RBC remaining in the lower portion were washed three times in iso-osmotic PBS by centrifugation at 515g for 10min to remove white cells and any traces of plasma. Following each spin, RBC were collected from the bottom layer of centrifugation tube (the buffy coat and the upper layer of RBC were discarded). The RBC were then resuspended in PBS at an approximate concentration of 1×10^6 RBC/mL (determined using a haemocytometer).

Various concentrations of the formulations to be tested were incubated with an appropriate quantity of RBC suspension (dependent on cell count) at 37°C for 1 hour. The incubation period was terminated via rapid high-speed centrifugation for 1min at 1960g. Percent haemolysis was determined by measuring the absorbance of the supernatant (released oxyhaemoglobin) at 540nm on a UV spectrophotometer (Unicam, Helios) and compared against positive control (RBC incubated in double-distilled water). Each test was performed in triplicate.

2.2.19 Small angle neutron scattering (SANS)

SANS (protocol adapted from Harvey *et al.*, 2005) measurements were performed on vesicles using a 12mm diameter neutron beam on the LOQ beam line at the ISIS pulsed neutron source (CCLRC Rutherford-Appleton Laboratory, Didcot, UK).

Liposome samples were prepared for SANS via the established hydration method described in Section 2.2.1. For each formulation composition investigated (except C₂₄PC preparations), three separate contrast experiments were performed: in the 1st study, the formulation was prepared and centrifuged in D₂O and fully-protonated phospholipids; in the 2nd, deuterated-chain lipid in H₂O; in the 3rd, fully-deuterated phospholipid in H₂O.

Preparations were placed in meticulously cleaned disc-shaped fused silica cells of path length 2mm. Using a 12mm diameter neutron beam, measurements were performed at 298K, with typically 40min exposure. Backgrounds from pure D₂O, were subtracted as appropriate.

2.2.20 Differential scanning calorimetry (DSC)

DSC was used to determine the thermotropic phase behaviour of mixed DSPC and cholesterol/tetradecanol bilayers. The instrument used was a Pyris Diamond DSC (Perkin Elmer, UK). Briefly, a volume containing 1-2mg of vesicle dispersion was placed on an aluminium pan and sealed. Empty sealed aluminium pan was used as the reference. The samples were scanned from -30 to 100°C at 2.5°C/min. The difference between both the pans was subsequently measured as a function of temperature. Data was analysed using Pyris Software (Version 5.0; Perkin Elmer, UK).

Chapter Three:

Solubilisation of drug within bilayer of vesicles: preliminary investigations

3.0 Introduction

Low solubility, and subsequent problems in bioavailability, has been recognised as one of the main culprits in many drug development failures (Perrie, 2005). However, poor aqueous solubility can often be circumvented by appropriate formulation work. Therefore, the development of colloidal models to overcome poor aqueous solubility of drug candidates from their chemical structure has attracted considerable attention. Whilst there is a large body of work investigating the application of colloidal surfactant vesicles (e.g. liposomes and niosomes) as carrier systems for the delivery of hydrophilic drugs (e.g. Carafa *et al.*, 1998 and Yoshioka *et al.*, 1994), their application as solubilising agents has received limited attention. However, these systems, due to the diversity in design, composition and construction which can be adopted, offer a dynamic and adaptable technology for enhancing drug solubility. To make effective use of these systems, the characteristics of these surfactant-based vesicles which favour bilayer drug loading and stability must be known. To support the systematic review of this various bilayer systems encapsulating model drug candidates can be used to aid in understanding and identifying the parameters(s) that enhance(s)/limit(s) solubility and can consequently supply valuable information to formulation scientists (Faller and Ertl, 2007).

The objectives of the work outlined within this chapter were to establish and validate an efficient method of determining encapsulation efficiency of MLV encapsulating poorly water soluble drugs without compromising accuracy and to establish a template formulation that can form the basis for further investigations. The intention of the initial series of investigations reported was therefore to:

- 1) Investigate the techniques and methods that can be used to quantitatively describe drug encapsulation in MLV;
- 2) Optimise technique used to determine encapsulation efficiency;
- 3) Determine the more efficient of two multilamellar systems considered, liposomes or niosomes;
- 4) Establish the effect of vesicle size on drug encapsulation.

As mentioned in Section 1.5, the poorly water-soluble model drugs investigated (summarised in Table 3.1) were as follows:

Drug	Molecular weight (Da)	Log P	Dissociation constant (pKa)	Solubility ($\mu\text{g/mL}$)	Overall nature of drug (functional group)
Diazepam	285	2.99	3.4	50	Basic
Ibuprofen	206	3.48	4.91	60 (Mohammed <i>et al.</i> , 2004)	Acidic
Midazolam	326	3.87	6.2 (Monzon <i>et al.</i> , 2001)	240	Basic
Phenytoin	252	2.24	8.33	22	Acidic
Propofol	178	3.84	11.1	153 (Altomare <i>et al.</i> , 2003)	Acidic
Rifampicin	833	3.72	1.7 & 6.8 (Pasquardini <i>et al.</i> , 2008)	Unknown	Acidic

Table 3.1 Summary of the characteristics of all model drug candidates investigated. Data obtained from Wishart *et al.* (2006) unless otherwise stated.

The concentration of the main lipid used to produce formulations was kept constant at $16\mu\text{mol}$ throughout as this has been used in the past (Gregoriadis *et al.*, 1999;

Mohammed *et al.*, 2004). Literature (Edwards and Baeumner 2006; Terzano *et al.*, 2005) express encapsulation in terms of 'percent encapsulation' which is typically defined as the total amount of encapsulant encapsulated MLV versus the total initial input of encapsulant. Percent mole per mole (% mol/mol) is another representation of entrapment efficiency which can also be found in publications (Edwards and Baeumner 2006; Gursoy *et al.*, 2004) and is defined as the molar amount of encapsulant per mole of lipid, calculated by dividing the concentration of encapsulant by the concentration of lipid. Results were initially presented in both forms for comparative purposes with the latter system primarily used thereafter.

3.1 Determination of drug entrapment within vesicles

This study describes the techniques and methods that can be used to quantitatively describe drug encapsulation in MLV with their respective advantages and limitations discussed where appropriate. Methods for determining the amount of material encapsulated within MLV typically rely on whether the bilayer is destructed with subsequent quantification of the released material, or if the bilayer is left intact and the incorporated drug is measured indirectly from quantification of the amount of drug not incorporated in a given medium such as PBS. The techniques used for analysis depend on the nature of the encapsulant and include spectrophotometry (Bangham *et al.*, 1965; Budai *et al.*, 2007; Mohammed *et al.*, 2007; Puglisi *et al.*, 1995), fluorescence spectroscopy (Guo *et al.*, 2003; Yoshioka *et al.*, 1994) and radio-labelled (Finlay and Wong, 1998; Proulx *et al.*, 2001) techniques.

Here, UV- and radiolabel-based techniques will be investigated as they have been used in the past for quantification of hydrophobic drugs (Mohammed *et al.*, 2004 and

Proulx *et al.*, 2001, respectively). The optimal method for the application is dependent on several factors, including ease of use/ interpretation and time consumption (Edwards and Baeumner, 2006).

To quantify drug loading into liposome formulations three different methods were employed:

- 1) UV analysis (as used in Budai *et al.*, 2007, Fatouros and Antisimiaris, 2002, and Puglisi *et al.*, 1995);
- 2) HPLC analysis (as described in Terzano *et al.*, 2005);
- 3) Radio-labelling (as used in Proulx *et al.*, 2001).

For liposome-loaded drug analysis, standard curves were prepared with known amounts of drug as described in Section 2.2.7. The calibration curves for respective drugs were prepared in 0.01M PBS (pH = 7.4) using appropriate concentrations in the ranges. For example, for calibration of midazolam, calibrators prepared in the range of 1-50µg/mL resulted in a R^2 value of 0.99.

3.1.1 UV analysis

Quantitative UV/visible analysis is often used to quantify the concentration of an analyte usually in an aqueous medium. In order to be able to do this, the analyte must absorb in the UV/visible region. Beer-Lambert's law is then used to determine its concentration (Pearce, 2006). UV/visible analysis is reported to provide a quick and efficient way of quantifying drug content (Gursoy *et al.*, 2004).

Diazepam and propofol loading of vesicles were determined by measuring the non-incorporated drug present in the supernatant, following separation of liposomes by centrifugation, using UV while the pellet was assessed using a more direct UV method established by Fatouros and Antimisariis (2002) to quantify liposomal loading of diazepam and propofol.

3.1.1.1 Assessment of supernatant

Drug concentration in the supernatant of liposomal suspensions after separation via centrifugation was examined by UV spectrophotometric analysis at respective wavelength (as determined via wave-scan of drug, with optimum peak selected). The quantity of entrapped drug into bilayer systems was determined indirectly following centrifugation and separation of the supernatant from the resulting pellet. The supernatant was assayed for the free drug and the amount of encapsulated drug into MLV was estimated after deducting the concentration of drug from the total amount of drug added initially (method described in detail in Section 2.2.3).

3.1.1.2 Assessment of liposomes

A method established by Fatouros and Antimisariis (2002) whereby the resulting pellet is spiked with isopropanol in a 50/50 v/v mixture and solubilised by vortexing, with analysis performed using UV was utilised to determine the quantity of drug (diazepam or propofol) encapsulated in liposomes.

The results (Tables 3.2 and 3.3) show that there was no significant difference between the results yielded via the direct method and those generated using the indirect assay for both diazepam and propofol. The encapsulation values of diazepam and propofol

in MLV liposomes via the indirect route (5.0 ± 0.8 and 20.3 ± 1.8 % mol/mol, respectively) were comparable to those seen for the more direct method (3.9 ± 0.3 and 19.0 ± 2.0 % mol/mol, respectively).

3.1.2 High performance liquid chromatography analysis

Drug concentrations were determined by using a HPLC system with UV detection as previously described (Terzano *et al.*, 2005). A known quantity of ibuprofen and midazolam were each added to a preparation of PC: cholesterol solution (0.89: 0.11 molar ratio) and centrifuged, with resulting pellet separated from supernatant. Thereafter, MLV suspension was lysed with isopropanol to, in theory, release all incorporated drug. Drug content in the pellet and the supernatant were measured using HPLC and compared against the theoretical supernatant drug concentration obtained via the indirect method by UV analysis (as described in Section 3.11).

Determination of ibuprofen and midazolam levels in the liposomes by HPLC following disruption of liposome bilayer gave a value of 15.6 ± 1.1 and 4.3 ± 0.4 % mol/mol, respectively. Analysis of the supernatant following centrifugation of the same batch of liposomes yielded an incorporation value of 13.7 ± 1.0 and 4.9 ± 0.8 % mol/mol, respectively. As shown in Table 3.3, the quantities of drug detected by HPLC and previously with UV (12.2 ± 2.0 and 4.8 ± 0.3 % mol/mol, respectively) gave approximately the same results. However, important differences between time taken and ease to perform analysis performed by HPLC and that performed by the UV assay were observed: UV analysis took significantly less time to complete as compared to analysis via HPLC.

3.1.3 Radio-labelling

Efficiency of drug entrapment in MLV by radioactivity measurements has been cited in the past (Finlay and Wong, 1998; Proulx *et al.*, 2001). For instance, Proulx *et al.* (2001) determined entrapment of 20(*S*)-Camptothecin (CPT), a chemotherapeutic agent used to treat solid tumours and leukaemias, in liposomes via the addition of small proportion of [³H]CPT as a radioactive tracer, thus reporting a successful route for determining MLV encapsulation of solute.

Here, indomethacin was selected for the duration of the study since its radioactive ¹⁴C isotope was readily available within our radioactive laboratory. MLV (PC: cholesterol, 0.89: 0.11 molar ratio) incorporating indomethacin was prepared as described in Section 2.2.6. MLV was spiked with radio-labelled [¹⁴C] indomethacin; the free drug was separated from the encapsulated drug by passing the MLV through a column pre-packed with Sephadex (G50). Fractions of 1.0mL aliquots were collected and the radioactivity of ¹⁴C within each aliquot was measured using a beta scintillation counter.

The encapsulation efficiency of indomethacin in MLV liposomes by radioactivity measurements was 7.1 ± 0.5 % mol/mol. This figure was not significantly different from the results (7.5 ± 0.4 % mol/mol; Table 3.3) obtained by using the UV-based method established by Bangham *et al.* (1965).

Validation results obtained (Sections 3.1.1, 3.1.2 and 3.1.3) revealed results obtained via the indirect method, where sample is centrifuged to remove precipitated species and the supernatant extracted for analysis, to be comparable to those obtained via the more direct route. The suitability of this has been previously demonstrated (Gursoy *et*

al., 2004; Mohammed *et al.*, 2004; Puglisi *et al.*, 1995). The UV method provides comparable results to HPLC with advantages of ease, speed, reproducibility, and consistent removal of precipitated MLV. It has been shown to be a sensitive method of detecting concentration of drug (Gursoy *et al.*, 2004). Thus, the indirect method was utilised for all further analysis for bilayer drug quantification.

From herein, for ease of comparison, results were expressed predominantly as percent mole per mole (% mol/mol) format.

Method	Encapsulation (%)				
	Diazepam	Ibuprofen	Indomethacin	Midazolam	Propofol
UV- supernatant	26.2 ± 4.3	39.1 ± 4.9	45.3 ± 4.5	27.5 ± 1.7	65.0 ± 5.7
UV- liposome	19.8 ± 1.5	n/a	n/a	n/a	61.0 ± 6.4
HPLC- supernatant	n/a	43.8 ± 2.5	n/a	28.8 ± 4.4	n/a
HPLC – liposome	n/a	46.3 ± 2.1	n/a	25.2 ± 2.6	n/a
Radio-labelling	n/a	n/a	42.8 ± 2.9	n/a	n/a

Table 3.2 Summary of drug loading by various methods used to determine entrapment. Encapsulation expressed in terms of 'percent encapsulation' which is typically defined as the total amount of encapsulant encapsulated MLV versus the total initial input of encapsulant. Results are expressed as the means of at least four experiments ± S.D.

Method	Encapsulation (% mol/mol)				
	Diazepam	Ibuprofen	Indomethacin	Midazolam	Propofol
UV- supernatant	5.0 ± 0.8	12.2 ± 2.0	7.5 ± 0.4	4.8 ± 0.3	20.3 ± 1.8
UV- liposome	3.9 ± 0.3	n/a	n/a	n/a	19.0 ± 2.0
HPLC- supernatant	n/a	13.7 ± 1.0	n/a	4.9 ± 0.8	n/a
HPLC – liposome	n/a	15.6 ± 1.1	n/a	4.3 ± 0.4	n/a
Radio-labelling	n/a	n/a	7.1 ± 0.5	n/a	n/a

Table 3.3 Summary of drug loading by various methods used to determine entrapment. Results expressed as percent mole per mole (% mol/mol). Results are expressed as the means of at least four experiments ± S.D.

3.2 Drug recovery from centrifugation method

To ensure that drug saturation levels were not exceeded in the solvent centrifugation, the drug recovery (and reproducibility) of six different compounds (diazepam, ibuprofen, midazolam, phenytoin, propofol and rifampicin) dissolved in PBS was

measured following centrifugation, as stated in Section 2.2.3. This was undertaken to ensure precipitation of drug within PBS did not occur. Such drug precipitation would result in precipitated drug sedimenting with the liposomes during centrifugation and hence giving artificially low concentrations of non-encapsulated drug.

Therefore to investigate this a known quantity of drug (1.00mg- as used in entrapment studies) was dissolved in the appropriate volume of PBS (i.e. the volume used during centrifugation; 60mL in case of diazepam; 30mL for all other drugs tested) so as not to exceed the threshold solubility values (Table 3.4) obtained from literature and centrifuged at 27, 200g for 30min. Recovery of drug within PBS was then analysed to ensure complete recovery of the drug within the supernatant.

Drug	Literature values for solubility in water ($\mu\text{g/mL}$)	Quantity of drug in supernatant (μg)	Percentage recovery (%)
Diazepam	50	960.0 ± 30.8	96.0 ± 3.1
Ibuprofen	60	943.7 ± 6.5	94.4 ± 0.7
Midazolam	240	933.3 ± 21.2	93.3 ± 2.1
Phenytoin	22	952.6 ± 18.7	95.3 ± 1.9
Propofol	153	924.3 ± 59.1	92.4 ± 6.0
Rifampicin	Unknown	944.4 ± 30.2	94.4 ± 3.0

Table 3.4 Recovery rate of 1.00mg drug dissolved in 30mL (or 60mL) of PBS, following centrifugation at 27,200g for 30min. UV analysis carried out at 212-268nm. Results are expressed as the means of three experiments \pm S.D.

The results obtained (Table 3.4) showed that a majority (~95%) drug recovery was obtained for all cases following the centrifugation process, suggesting a volume of 30mL (60mL PBS with respect to diazepam) was sufficient to dissolve 1.00mg of respective drug. No loss of free drug was seen indicating that drug saturation is not exceeded at above mentioned volumes.

3.3 Effect of centrifugation

The method used to analyse drug encapsulation involved the separation (washing) and photometric analysis of the 'free' drug. This method offered the quickest throughput of formulations. However, it is essential to ensure that majority, if not all unentrapped and surface adsorbed drug is removed from the drug-loaded vesicles. The objective of this study was to determine the number of washings that would be satisfactory to remove majority of the unincorporated drug and also indicate if repeated centrifugation promoted continued drug loss from the formulations.

The influence of repeat washings was examined on a selective batch of formulations (DMPC or DSPC: cholesterol, 0.89; 0.11 molar ratio) to optimise the removal of non-incorporated drug. The formulations were each centrifuged three times, with each resulting supernatant separated for drug-encapsulation analysis via UV at appropriate wavelengths. Two drugs midazolam (240 μ g/mL) and diazepam (50 μ g/mL) were investigated as they offered the highest and one of the lowest solubility ranges of the drugs under investigation (Table 3.4). DMPC and DSPC formulations were used as they were used in several subsequent experiments.

The results (Figures 3.1 and 3.2) obtained show that a small quantity of drug (~1-5%; midazolam or diazepam) is present following the second centrifugation of the formulations investigated and that only minimal amounts (2-3%) are present after the third washing and is independent of type of lipid utilised for formulation. This suggests that reliable indications of drug entrapment can be achieved by performing two centrifugations of the MLV/drug suspension mixture. Consequently, two centrifugations were used for all further formulation studies involving the encapsulation and subsequent analysis of drug.

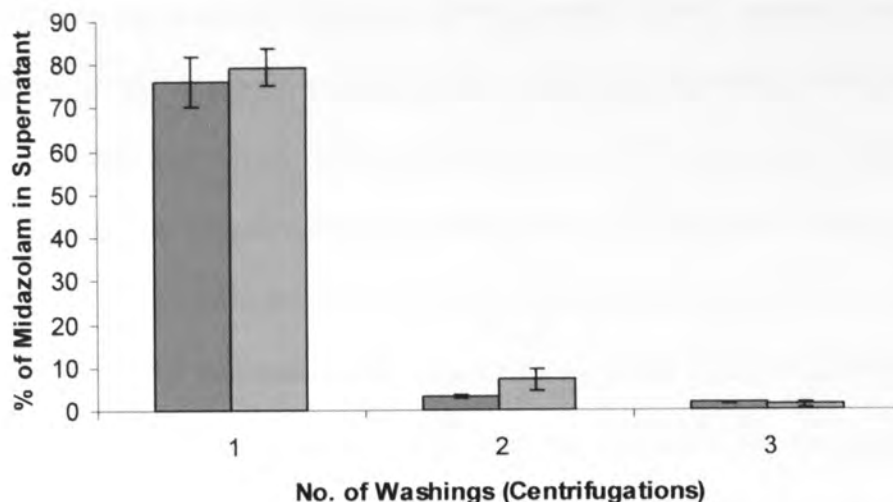


Figure 3.1 The influence of repeat centrifugations on DMPC: cholesterol (purple bars) and DSPC: cholesterol (blue bars) formulations (0.89: 0.11 molar ratio) with midazolam encapsulation at 258nm. Values represent percentage of initially introduced drug and are expressed as the means of three experiments \pm S.D.



Figure 3.2 The influence of repeat centrifugations on DMPC: cholesterol (purple bars) and DSPC: cholesterol (blue bars) formulations (0.89: 0.11 molar ratio) with diazepam encapsulation at 228nm. Values represent percentage of initially introduced drug and are expressed as the means of three experiments \pm S.D.

3.4 Lipid and non-ionic surfactant based vesicles

Liposomes are a well studied colloidal carrier system, though they have some fundamental problems, such as chemical instability and complications in mass production (Sinha *et al.*, 2002). Thus, to eliminate such limitations, niosomes were

examined as an alternative drug delivery system. These carrier systems are formulated from non-ionic surfactants that are biodegradable, inexpensive and relatively stable (Sinha *et al.*, 2002; Yoshioka *et al.*, 1994). A literature search found few articles that examined niosomal encapsulation of hydrophobic drugs and even fewer that directly comparing liposomes and niosomes with respect to encapsulation of these drugs. As a consequence, a comparative study was performed between commonly investigated liposome and niosome formulations to evaluate the performance of these formulations in terms of drug encapsulation as well as size and zeta potential. Ibuprofen, which is a weak acidic molecule with a pKa of 4.9 and a partition coefficient value of 3.5, was selected as a model drug candidate to allow comparison to previous studies (Mohammed *et al.*, 2004).

3.4.1 Liposomal ibuprofen incorporation

Drug loading within phosphatidylcholine based multilamellar liposomes was investigated and the influence this had on the liposome mean volume diameter and zeta potential was initially studied. Liposomes were formulated using PC (16 μ mol) and cholesterol (4 μ mol) with and without 4 μ mol of the negatively charged dicetyl phosphate (DCP). The cholesterol content was chosen based on previously reported studies (Mohammed *et al.* 2004). Whilst the inclusion of a negatively charged component within the liposomes was reported to reduce ibuprofen loading (Mohammed *et al.*, 2004) this was investigated to allow subsequent comparison with the non-ionic based vesicles which require DCP for effective formulation (Uchegbu and Florence, 1995).

Table 3.5 shows that the presence of a negatively charged lipid influences the surface

charge, with the zeta potential of the vesicles decreasing from a near neutral to -71mV after the addition of 14 total molar % DCP. This change in bilayer composition also resulted in a significant reduction in ibuprofen loading for 7.49 % mol/mol to only 2.39 % mol/mol when DCP was present ($P < 0.05$) (Table 3.5), however, addition of DCP did not significantly affect the vesicle size compared to formulation without the charged compound. Previous studies by Puglisi *et al.* (1995) and Mohammed *et al.* (2004) have shown that the presence of negatively charged lipids can effect the encapsulation of the bilayer incorporated drugs ofloxacin and ibuprofen, respectively. Since both of these drugs possess anionic groups within their structure, the reduction of drug loading may be due to electrostatic repulsive effects generated from interactions between negatively charged head-group of DCP and the slightly anionic ibuprofen resulting in the reduced uptake of the drug into the liposome bilayer (Mohammed *et al.*, 2004). However, Bhatia (2004) observed that the inclusion of DCP did not influence bilayer encapsulation of the poorly water-soluble anti-oestrogen drug tamoxifen, a drug that comprises a slightly anionic structure. The presence or absence of ibuprofen within the liposomes made no significant difference to the zeta potential of the liposomes (Table 3.5).

Formulation	Empty MLV		Ibuprofen-loaded MLV		
	Size (μm)	ZP (mV)	Size (μm)	ZP (mV)	Encapsulation (% mol/mol)
PC: cholesterol (0.67: 0.33 molar ratio)	4.7 ± 1.6	0.1 ± 1.4	5.0 ± 1.2	-0.9 ± 0.2	7.5 ± 1.6
PC: cholesterol: DCP (0.57: 0.29: 0.14 molar ratio)	4.5 ± 1.4	-70.8 ± 10.0	4.7 ± 0.5	-70.1 ± 4.4	2.4 ± 0.5

Table 3.5 Characteristics of liposomes composed of PC and cholesterol with and without the negatively charged DCP. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. The ibuprofen loading of MLV was monitored as previously described in Section 2.2.3. The drug content was analysed by UV spectroscopy at 221nm wavelength. Results are expressed as the means of four independent experiments \pm S.D.

3.4.2 Niosomal ibuprofen incorporation

The above basic liposome formulation was then compared to non-ionic surfactant based vesicles, niosomes, which can offer a cheaper and more stable alternative to its phospholipid-based systems (Yoshioka *et al.*, 1994). Niosomes also have numerous other advantages over liposome systems such as the greater convenience in storage of its main ingredients (Uchegbu and Vyas, 1998) and vesicle aggregation and fusion normally occur at a significantly lower rate (Carafa *et al.*, 1998). Furthermore, the non-ionic nature of the structures renders them to be less toxic than vesicles formed from their ionic counterparts therefore potentially proposing as hopeful vehicles for drug delivery (Florence and Attwood, 1998). Sorbitan esters (Figures 3.3 and 3.4) were selected as the non-ionic surfactants to be investigated. They are commercial products that are generally insoluble in water and are utilised as water-in-oil emulsifiers and as wetting agents (Florence and Attwood, 1998). The major esters are listed in Table 3.6 along with their respective hydrophile-lipophile balance (HLB).

Niosomes of sorbitan esters have been synthesised previously: Varshosaz *et al.* (2003) successfully synthesised insulin-loaded niosomes using the hydration method (Bangham *et al.*, 1965) from Span 20, 40, 60 and 80 with the inclusion of cholesterol, while Uchegbu *et al.* (1996) cite the successful production of Span 60 niosomes encapsulating the water-soluble drug, doxorubicin. Studies carried out by Manosroi *et al.* (2003) conclude that MLV formulated from the longer alkyl chain (C_{18}) surfactants give higher entrapment efficiency of water-soluble substances and displayed greater stability than those yielded from the shorter alkyl chain (C_{12}) non-ionic surfactants.



Illustration removed for copyright restrictions

Figure 3.3 Sorbitan monoester vesicle-forming surfactants. *A* = sorbitan monolaurate (Span 20), *B* = sorbitan monopalmitate (Span 40), *C* = sorbitan monostearate (Span 60). *D* = sorbitan monooleate (Span 80) (Uchegbu and Florence, 1995).

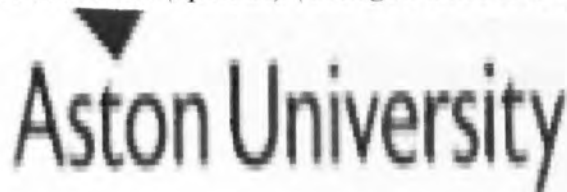


Illustration removed for copyright restrictions

Figure 3.4 Sorbitan triester vesicle forming surfactant. *E* = sorbitan trioleate (Span 85) (Uchegbu and Florence, 1995).



Illustration removed for copyright restrictions

Table 3.6 List of non-ionic sorbitan esters along with their respective HLB (hydrophile-lipophile balance) values (Florence and Attwood, 1998).

3.4.2.1 Non-ionic surfactant vesicles versus phospholipid-based vesicles

Initially, aims were to formulate niosomes with and without the inclusion of DCP, however subsequent studies revealed Span 60 niosomes could not be effectively

formed without the presence of DCP within the bilayer. This could be as a result of destabilisation leading to vesicle aggregation as reported in a review by Uchegbu and Vyas (1998). Therefore consequently only niosomes supplemented with 5-14 total molar % DCP were prepared. Span 60 niosomes were formulated at two different ratios of surfactant: cholesterol, 2:1 and 1:1, to investigate the influence of cholesterol on ibuprofen encapsulation, vesicle size and zeta potential.

The results in Table 3.7 demonstrate the substitution of PC for Span 60 results in the zeta potential of the empty vesicles (both containing 14.3 total molar % DCP) changing from -71mV to -50mV, respectively. Upon bilayer loading of ibuprofen, no further significant difference in the surface charge was observed. The substitution of PC for the non-ionic surfactant Span 60 resulted in a significant reduction in drug incorporation, when both prepared with 14.3 total molar % DCP. The lower drug encapsulation for the non-ionic surfactant-based vesicles could likely be due to lower integrity of the spans (Agarwal *et al.*, 2001). As expected, upon decreasing the concentration of DCP from 14.3 to 5.0 total molar % of empty Span 60 MLV, the zeta potential significantly became less negative (by approximately 15mV). However, this reduction in DCP did not significantly affect drug entrapment efficiency. Vesicle sizes between the MLV liposomes and niosomes (as well as between empty and loaded) were not significantly different with sizes around 4.5 - 5.1 μ m being measured for all formulations.

The entrapment efficiency of vesicles formulated using Span 60 and cholesterol (and DCP) in a 1:1 ratio was not significantly different from those formulated at a ratio of 2:1. This is in contrast to the results published by Agarwal *et al.* (2001) who showed

that the liposomal loading of the poorly water soluble drug dithranol was enhanced when PC and cholesterol are in equal molar ratio, while previous studies by Santucci *et al.* (1996) concluded that if the non-ionic surfactant: cholesterol ratio was above or below 1:1, it was very difficult to prepare vesicles. Furthermore, Yoshioka *et al.* (1994) prepared Span 60 niosomes without cholesterol which subsequently formed a gel and only on the addition of cholesterol was a homogenous niosome dispersion obtained, thus probably displaying the stabilising features of the lipid.

Formulation	Empty MLV		Ibuprofen-loaded MLV		
	Size (μm)	ZP (mV)	Size (μm)	ZP (mV)	Encapsulation (% mol/mol)
PC: cholesterol: DCP (0.57: 0.29: 0.14 molar ratio)	4.5 ± 1.4	-70.8 ± 10.0	4.7 ± 0.5	-70.1 ± 4.4	2.4 ± 0.5
Span 60: cholesterol: DCP (0.57: 0.29: 0.14 molar ratio)	4.7 ± 0.3	-50.2 ± 6.4	4.5 ± 0.3	-49.7 ± 5.5	1.0 ± 0.3
Span 60: cholesterol: DCP (0.48: 0.48: 0.05 molar ratio)	6.8 ± 0.1	-35.8 ± 6.4	8.5 ± 0.1	-44.3 ± 1.8	1.5 ± 0.3

Table 3.7 Characteristics of vesicles composed of PC, cholesterol and 14 total molar % DCP and of vesicles formulated using a sorbitan monoester (Span 60), cholesterol, and 5-14 total molar % DCP. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9. The ibuprofen loading of MLV was monitored as previously described in Section 2.2.3. The drug content was analysed by UV spectroscopy at 221nm wavelength. Results are expressed as the means of four independent experiments \pm S.D. (Data for PC formulation previously presented in Section 3.4.1, is presented here for ease of comparison)

3.4.2.2 The effect of hydrophobic chain length of non-ionic surfactant

To investigate the effect of the hydrophobic chain length on surfactants niosomes formulated with Span 20, 40, 80 and 85 (with the inclusion of DCP) and at a ratio of 1:1 surfactant: cholesterol ratio was further examined. Five different formulations were prepared as- Span X (47.5 total molar %): cholesterol (47.5 total molar %): \pm DCP (5.0 total molar %), where X= 20, 40, 60, 80 and 85.

3.4.2.2.1 Encapsulation

The results generated (Table 3.8) illustrate that niosomes formed from Span 60 yield the highest ibuprofen-encapsulation rate (1.51 ± 0.34 % mol/mol) with encapsulation efficiency following the trend Span 60 (C_{18}) > Span 40 (C_{16}) \geq Span 20 (C_{12}) vesicles ($P < 0.05$). This correlates with past studies: Ong and Manoukian (1988) investigated the solubilisation of timobesone acetate, a poorly water-soluble corticosteroid employed in inflammatory therapy, in solutions of non-ionic surfactants. They discovered that the capacity to solubilise the compound increased with increasing length of the hydrophobic tail of the surfactants. Consequently, timobesone was assumed to be solubilised in the micellar hydrophobic core. The results obtained reflect the influence of the phase transition temperature. The non-ionic surfactant having the highest phase transition temperature (Span 60) provided the highest ibuprofen-loading rate. This is probably due to the phase transition temperature being dictated by the length of the hydrophobic tail of the Span molecules. As Span 60 has the longest hydrophobic alkyl chain group, it would be expected that there will be more hydrophobic area available between the membranes for ibuprofen to interact (Mohammed *et al.*, 2004).

Another explanation could be attributed to perhaps the longer chain lengths yielding stronger niosome bilayers (reflected by higher incorporation of hydrophilic drugs as reported by Manosroi *et al.*, 2003 and Yoshioka *et al.*, 1994) and therefore these bilayers may also be better suited to load the hydrophobic drug into.

Spans 80 and 85 have the same alkyl chain length (C_{18}) as Span 60, but vesicles could not completely be separated from untrapped ibuprofen as an incomplete pellet formation was visible following centrifugation at 27,200g for 30min.

3.4.2.2.2 Vesicle size

With regard to MLV size, increasing alkyl chain length from C_{12} to C_{14}/C_{16} of the surfactant molecule resulted in significantly larger vesicles with and without ibuprofen-entrapment; drug- loading enhanced the diameter of the niosomes (Table 3.8).

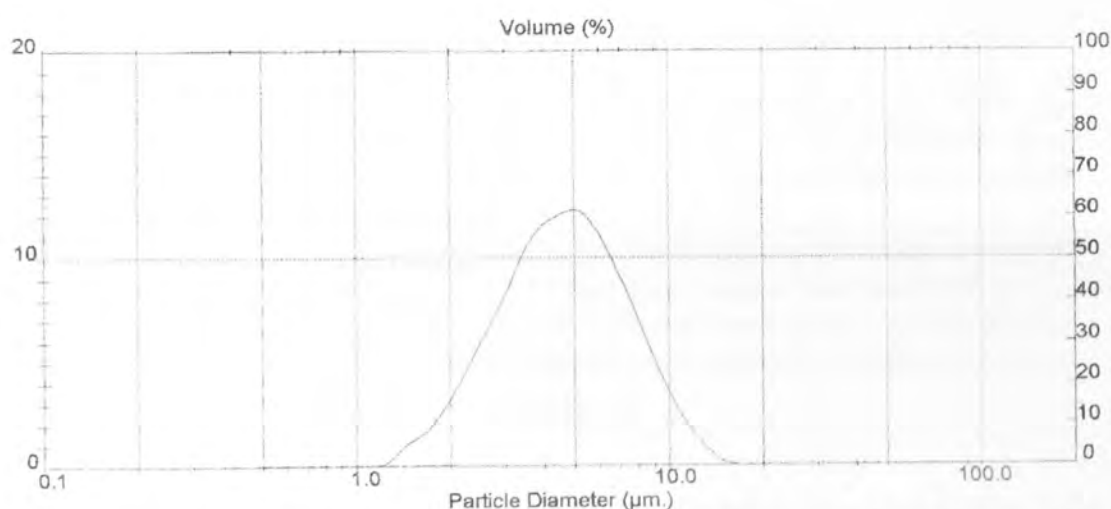


Figure 3.5 Particle size distribution profile of a liposomal formulation (PC: cholesterol, 0.67: 0.33 molar ratio). The mean volume diameter of vesicles was determined as described in Section 2.2.8.

Figure 3.5 reveals a scan for a PC: cholesterol formulation (0.67: 0.33 molar ratio) with a mean vesicle diameter of 5.02μm, with 90% of the liposomal population equal or below 13.1μm. The size range for the MLV formulations prepared in this section was generally found to be within this range. This scan, therefore, generally represented the normal size distribution of all the vesicles examined in this section. As a result, the span was not reported again for all subsequent studies.

3.4.2.2.3 Vesicle zeta potential

In terms of the charge of the vesicles, it generally remained unchanged following drug-encapsulation, suggesting that the alkyl chain length of the non-ionic surfactants does not influence the bilayer surface charge (Table 3.8).

Formulation	Empty MLV		Ibuprofen-loaded MLV		
	Size (μm)	ZP (mV)	Size (μm)	ZP (mV)	Encapsulation (% mol/mol)
Span 20: cholesterol: DCP (0.48: 0.48: 0.05 molar ratio)	5.8 ± 0.2	-44.5 ± 3.7	6.4 ± 0.1	-40.2 ± 2.2	0.6 ± 0.1
Span 40: cholesterol: DCP (0.48: 0.48: 0.05 molar ratio)	6.5 ± 0.1	-55.3 ± 12.4	8.0 ± 0.6	-55.1 ± 13.9	0.7 ± 0.2
Span 60: cholesterol: DCP (0.48: 0.48: 0.05 molar ratio)	6.8 ± 0.1	-35.8 ± 6.4	8.5 ± 0.1	-44.3 ± 1.8	1.5 ± 0.3

Table 3.8 The effect of alkyl chain length on niosomal ibuprofen loading, MLV size and zeta potential. Niosomes formulated using a sorbitan monoester (Span 20, 40 or 60), cholesterol and DCP in a total lipid content of $50\mu\text{mol}$. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. The ibuprofen loading of MLV was monitored as previously described in Section 2.2.3. The drug content was analysed by UV spectroscopy at 221nm wavelength. Results are expressed as the means of four experiments \pm S.D. (Data for Span 60 formulation previously presented in Section 3.4.2.1, is presented here for ease of comparison)

Overall this work shows that ibuprofen encapsulation for the niosomal formulations tested was inferior to drug-loading by their liposomal analogue (Table 3.7). This finding agrees with results obtained by one of the few publications obtained following an extensive literature search for comparative studies of the two systems with regards to entrapment of a hydrophobic drug; investigation by Agarwal *et al.* (2001) into these systems as solubilisation agents for dithranol reported low vesicular drug incorporation and a quicker rated drug release by non-ionic surfactant based vesicles when weighed against their liposomal counterparts although Fang *et al.* (2001) found no significant differences between liposomes and niosomes in the

encapsulation of enoxacin, a poorly water-soluble antibiotic. The increased entrapment by liposomal bilayers may be due to the enhanced availability of lipophilic nature (one phospholipid molecule has two alkyl chains compared to the one pertaining to non-ionic surfactants), which can now accommodate the drug molecules to a higher degree (Agarwal *et al.*, 2001). Although niosomes are understood to alleviate drawbacks associated with liposomes (Sinha *et al.*, 2002; Yoshioka *et al.*, 1994), on the basis of results obtained, hereafter the lipid based systems were further investigated.

3.5 The effect of liposome morphology on drug loading within liposomes

Results obtained in the previous section, established phospholipid-based vesicles as most efficient (compared to non-ionic surfactant based versions) at incorporating a poorly water-soluble drug. The subsequent stage was to establish whether the lamellarity of the vesicle will have a profound effect on drug encapsulation figures. Multilamellar vesicles (harbouring multiple bilayers parallel to one another enclosing an aqueous core; Figure 3.6 and Figure 3.7) were compared to small unilamellar vesicles (SUV; vesicles consisting of a single bilayer enclosing an aqueous core; Figure 3.6).

Indomethacin was selected for the duration of the study since its radioactive ^{14}C isotope was readily available within our laboratory. MLV and SUV were prepared as described in Sections 2.2.1 and 2.2.2, respectively. Liposomes were spiked with radio-labelled [^{14}C] indomethacin; the free drug was separated from the entrapped drug by passing the vesicles through a column pre-packed with Sephadex (G50). Fractions of 1.0mL aliquots were collected and the radioactivity of ^{14}C within each

aliquot was measured (using a beta scintillation counter) in the liposome aliquots and compared with the total radioactivity of labelled indomethacin added.

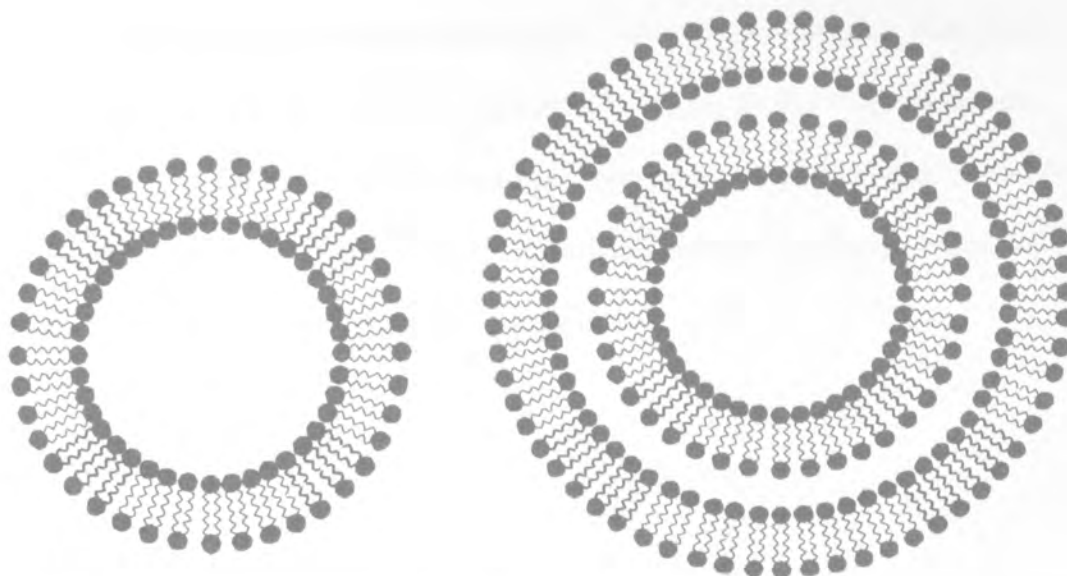


Figure 3.6 Illustration representing small unilamellar vesicles (between 15nm and 100nm in size; left) and multilamellar vesicles (between 100nm and 10 μ m; right).

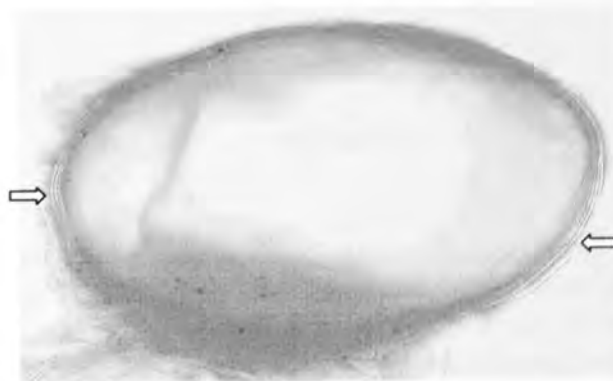


Figure 3.7 A transmission electron micrograph of a drug-entrapped formulation prepared from 16 μ mol DSPC with 11 total molar % cholesterol content. Image displaying the multiple bilayers of liposomes. Image was taken using a JEOL 1200EX microscope fitted with a LaB6 filament, with an operating voltage of 40-120kV.

MLV (spiked with radioactive [14 C] indomethacin) were subjected to a Sephadex G50 gel column (Process 1) which yielded two peaks, with the first lying between aliquot numbers 3 and 10 and the subsequent peak appearing between 14 and 21 (Figure 3.8).

Using gel exclusion chromatography, molecules can be separated by their size by chromatography on columns of beads of gel that constitute of small pores allowing smaller molecules to spend more time within the pores of the support medium, and therefore move relatively more slowly compared with larger molecules. Therefore, on the assumption that liposomes are significantly larger in size than indomethacin molecules, it was accepted that the first peak represented that of the vesicles and the latter corresponding to the free drug. Using this approach, an entrapment efficiency of $42.8 \pm 2.9\%$ was observed for the MLV formulation.

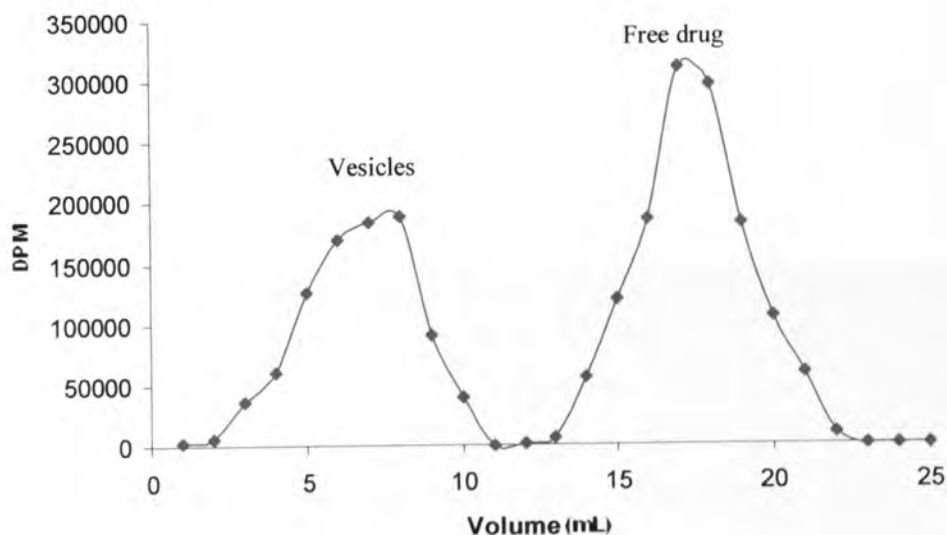


Figure 3.8 Investigating 'unwashed' MLV encapsulating radio-labelled ^{14}C indomethacin. The labelled drug was incorporated in MLV composed of PC and cholesterol ($16.2\mu\text{moles}$). The vesicles were separated from the un-entrapped indomethacin by column separation. Samples were collected intermittently (1mL) and analysed using a beta scintillation counter. The graph is a representation of results generated from three experiments.

Fresh sample of MLV prepared (in the presence of 1.0mg indomethacin spiked with radioactive indomethacin) were subjected to sonication to produce SUV that were generally around 170nm in diameter. The SUV were passed down the Sephadex G50 column (Process 2) with aliquots collected in 1.0mL fractions. Results of the gel

chromatography again yielded two distinct regions, first between fraction numbers 4 and 10 (that of indomethacin-loaded SUV) and the second peak of free indomethacin between 13 and 20 (Figure 3.9). An average SUV encapsulation rate (based on the amount of drug added prior to sonication) of $37.5 \pm 3.0\%$ was obtained. There was no significant difference between the SUV encapsulation and the one observed in case of MLV ($42.8 \pm 2.9\%$; Table 3.9).

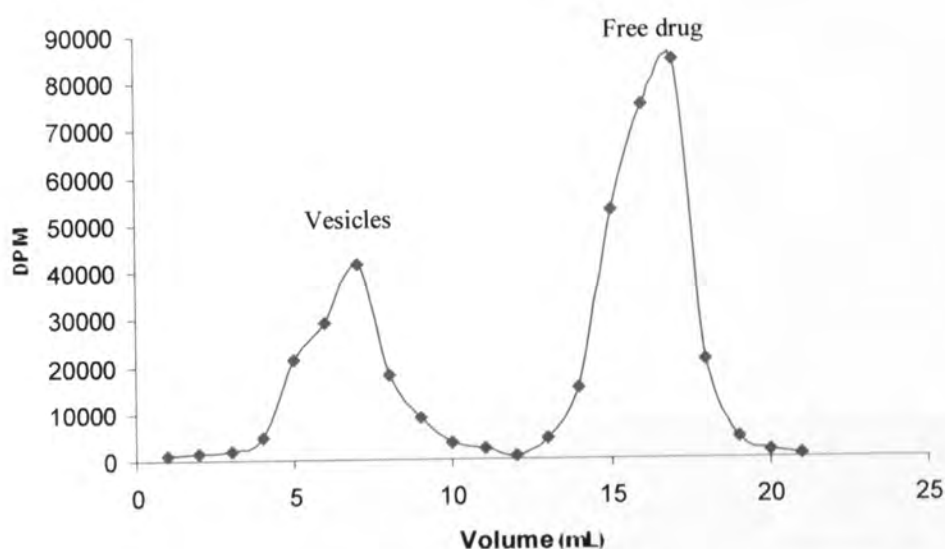


Figure 3.9 Investigating ‘unwashed’ SUV encapsulating radio-labelled ^{14}C indomethacin. The labelled drug was incorporated in SUV composed of PC and cholesterol (0.89:0.11 molar ratio). The vesicles were separated from the free drug via column separation. Samples were collected intermittently (1mL) and analysed using beta scintillation counter. The graph is a representation of results generated from three experiments.

To investigate if the presence of free drug in the suspension influenced drug loading in SUV the experiment was repeated with MLV in which free drug had been removed prior to sonication (via centrifugation), thus yielding ‘washed’ SUV, and passed down a Sephadex G50 column (Process 3). Results are presented in Figure 3.10 and summarised in Table 3.9. Interestingly, as shown in Table 3.9, removal of free drug from the MLV suspension prior to sonication made no significant difference ($35.2 \pm$

2.1% vs. $37.5 \pm 3.0\%$ for 'washed' SUV vs. 'unwashed' SUV, respectively) in the drug loading measured (% entrapment of SUV based on the amount of drug present prior to sonication, i.e. MLV loading) for SUV. This suggests that concentration gradient created when un-encapsulated indomethacin is separated from bilayer-loaded vesicles prior to sonication in Process 3 (Figure 3.11) has no effect on drug loading.

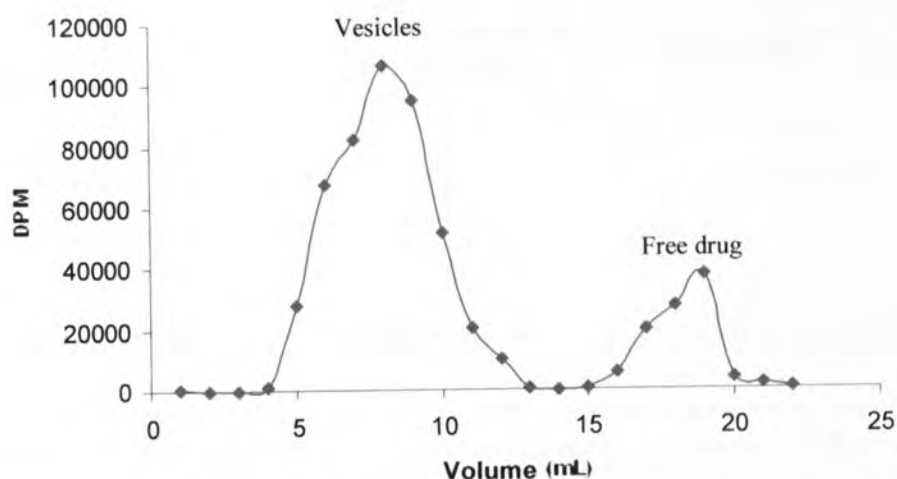


Figure 3.10 Investigating 'washed' SUV encapsulating radio-labelled ^{14}C indomethacin. The labelled drug was incorporated in SUV composed of PC and cholesterol (16:2 μmoles). The vesicles were separated from the un-encapsulated drug via column separation. Samples were collected intermittently (1 mL) and analysed using a beta scintillation counter. The graph is a representation of results generated from three experiments.

Method	Encapsulation (%)	Size (nm)
MLV ('Unwashed')	42.8 ± 2.9	5257 ± 29
SUV (From 'un-washed' MLV)	37.5 ± 3.0	149 ± 19
SUV (From 'washed' MLV)	35.2 ± 2.1	173 ± 21

Table 3.9 A summary of results for liposomal (PC: Cholesterol, 0.89:0.11 molar ratio) encapsulation of ^{14}C radio-labelled indomethacin comparing 'washed' SUV and MLV against 'un-washed' SUV. Analysis carried out on a beta scintillation counter. Results are expressed as the means of three experiments \pm S.D.

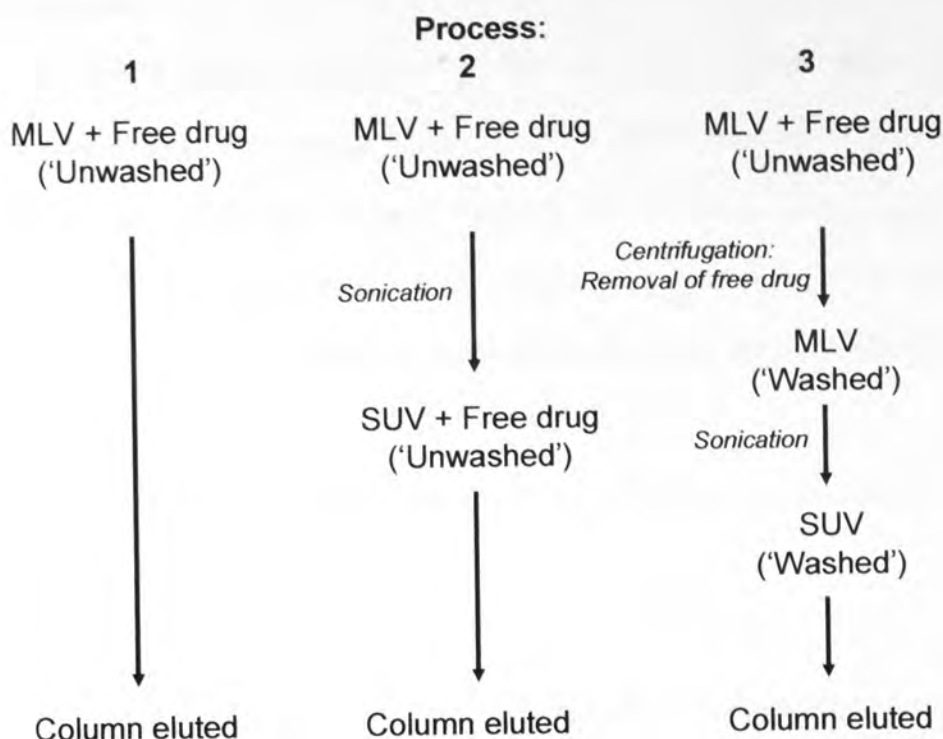


Figure 3.11 Schematic illustration of the three gel chromatography processes as discussed above- Process 1: MLV prepared subjected to a Sephadex G50 gel column; Process 2: Fresh sample of MLV subjected to sonication to produce SUV, then passed down the Sephadex G50 column; Process 3: Free drug removed from MLV prior to sonication, thus yielding ‘washed’ SUV, and passed down a Sephadex G50 column.

The three processes are summarised in Figure 3.11. Overall this suggests that lipid: drug ratio is probably more important than the morphology of the preparation. Therefore, for all further liposomal studies, the larger MLV (as opposed to SUV) were prepared and examined for solubilisation of poorly water-soluble drugs. Since one of the objectives of this work was to investigate the role of liposome composition on the drug loading MLV which are simple to produce and boast a relatively rugged structure were used in subsequent studies. SUV, whilst potentially more biologically relevant for several routes of delivery, the processing time required in the production of SUV via the use of a probe sonicator and determining drug encapsulation values is considerably longer (Kirby and Gregoriadis, 1999).

3.6 Conclusions

Validation results obtained revealed data obtained via the indirect method, where sample is centrifuged to remove precipitated species and the supernatant extracted for analysis, to be comparable to those obtained via the more direct routes. The advantages of this technique include ease, speed and reproducibility. As a result, the indirect method was utilised for all further analysis for bilayer drug quantification.

From herein, for ease of comparison, results were expressed predominantly as percent mole per mole (% mol/mol) units.

To ensure that drug saturation levels were not exceeded in the solvent centrifugation, the drug recovery (and reproducibility) of six poorly water-soluble drugs dissolved in aqueous media was measured following centrifugation. The UV measurements recorded showed that a majority drug recovery for all cases following the centrifugation process, suggesting a volume of 30mL (60mL PBS with respect to diazepam) was sufficient to solubilise 1.00mg of respective drug. Furthermore, two centrifugations of the MLV/drug suspension mixture were found to be sufficient to separate majority of untrapped drug.

Data collated revealed that ibuprofen encapsulation for the niosomal formulations tested was significantly lower than by their liposomal analogue possibly as a result of increased availability of lipophilic regions with bilayers prepared from ionic surfactants. Liposomes were therefore further investigated from herein.

Investigating the effect of size/lamellarity of vesicle on drug encapsulation efficiency, lipid: drug ratio was revealed to be possibly the more important factor compared with morphology of the preparation. Therefore, for all further liposomal studies, the larger MLV (as opposed to SUV) were prepared and examined for solubilisation of poorly water-soluble drugs.

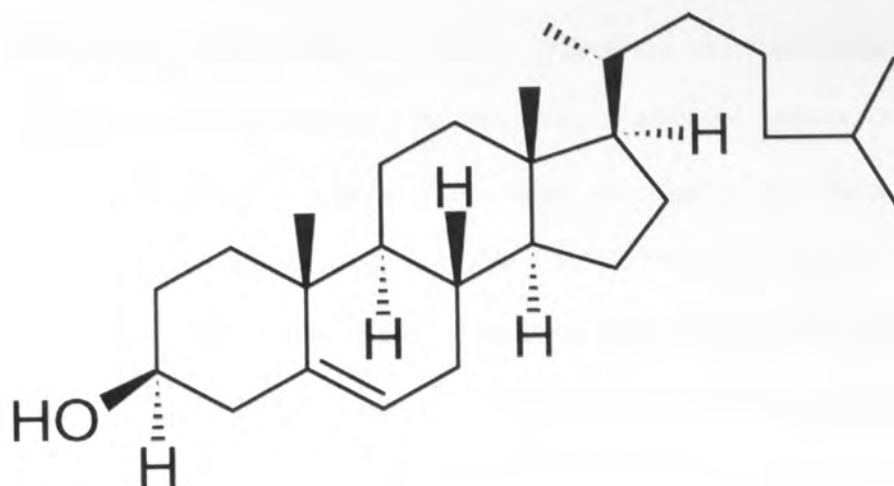
Chapter Four:

The effect of cholesterol within the bilayer constructs

4.0 Introduction

The efficient entrapment of a given substance is a key condition for successful application of liposomes (Felnerova *et al.*, 2004). To achieve the most favourable bilayer conditions for the optimum encapsulation of hydrophobic model drug candidates, it has been reported to be dictated by a combination of lipid characteristics- for example lipid alkyl chain moiety and head-group charge (Mohammed *et al.*, 2004) as well as drug properties, such as molecular weight and Log P (Fatouros and Antimisiaris, 2002).

The physical properties of liposomes are closely associated with the specific arrangement and distribution of the components within the phospholipid bilayer. One such component to exist in abundance in mammalian plasma membranes is cholesterol (Ohtake *et al.*, 2005). Cholesterol (Figure 4.1) plays a key role in animal and fungal cell membranes as a modulator of physical and functional properties of lipid bilayers (Gallova *et al.*, 2004) and is therefore widely accepted to influence the bilayer stability of liposomes (Devaraj *et al.*, 2002; Manosroi *et al.*, 2003). It is also recognised to influence bilayer fluidity (Puglisi *et al.*, 1995) and permeability of drugs (Hac-Wydro and Wydro, 2007; Vilcheze *et al.*, 1996). At high concentrations (> 30 total molar %), the substance can totally eliminate phase transition and reduce the fluidity of membranes at a temperature greater than the T_c , which enhances liposomal stability while rendering them less leaky following systemic administration (Sharma and Sharma, 1997). A balance between the three parameters, stability, rigidity and permeability, may therefore be essential in order to achieve optimum encapsulation efficiency of a lipophilic moiety.



Molecular weight: 386.7

Molecular formula: C₂₇H₄₆O

Hydrophilic moiety: -OH

Hydrophobic regions: Hydrocarbon 'rings' and 'tail'

Figure 4.1 *Molecular structure and properties of cholesterol.*

4.1 The effect of liposomal cholesterol content on liposomal systems

4.1.1 Poorly soluble drug loading

To achieve the most favourable bilayer conditions for a hydrophobic drug to encapsulate, the cholesterol concentration was optimised using PC. In these studies the drugs diazepam, ibuprofen, midazolam and propofol were investigated. Various concentrations of cholesterol, ranging from 0 to 33 total molar %, were investigated whilst keeping the concentration of the phospholipid constant (16 μ mol). A drug quantity of 1.00mg was added to each formulation and encapsulation figures determined as relative percentage to the total stated.

Concerning the effect of cholesterol concentration on the encapsulation efficiency of drug in the prepared liposomes, results showed that the encapsulation efficiency of drug was generally decreased by increasing cholesterol content (Figure 4.2). For propofol this is most notable with a linear decrease in propofol entrapment with increase in cholesterol concentration ($P < 0.05$). Of those tested (0, 11, 20, 33 total molar %), sample without any cholesterol encapsulated the highest quantity of propofol (23.9 ± 1.5 % mol/mol) (Figure 4.2). A parallel trend for formulations encapsulating midazolam and diazepam can be seen (Figure 4.2). For ibuprofen loading, whilst there is an overall trend apparent, there was no significant difference in ibuprofen entrapment between the formulation containing no cholesterol and the MLV containing 11 or 20 total molar % cholesterol. However, increasing the cholesterol content to 33 total molar % resulted in significant reduction ($p < 0.05$) in drug incorporation to 7.5 ± 1.6 % mol/mol. Overall the encapsulation rate of vesicles formulated without the addition of cholesterol was significantly higher than that of the formulation containing 33 total molar% cholesterol ($P < 0.05$).

The trend of decreasing drug encapsulation with increasing levels of cholesterol inclusion could be attributed to the enhancement of the overall rigidity of the bilayer and gradual reduction in bilayer permeability to small hydrophilic solutes and ions (Devaraj *et al.*, 2002). Gregoriadis and Davis (1979) revealed that the presence of 50 total molar % cholesterol within a MLV formulation enhanced formulation stability whilst reducing the permeability of the bilayers. There could also exist a physical effect where a boost in the presence of cholesterol could result in a competition for packing space, therefore excluding drug as the phospholipids assemble into vesicles

(Mohammed *et al.*, 2004). The bulky nature of cholesterol, when entrapped in the lipid bilayer, decreases the mobility of hydrophobic tails (Deol and Khuller, 1997).

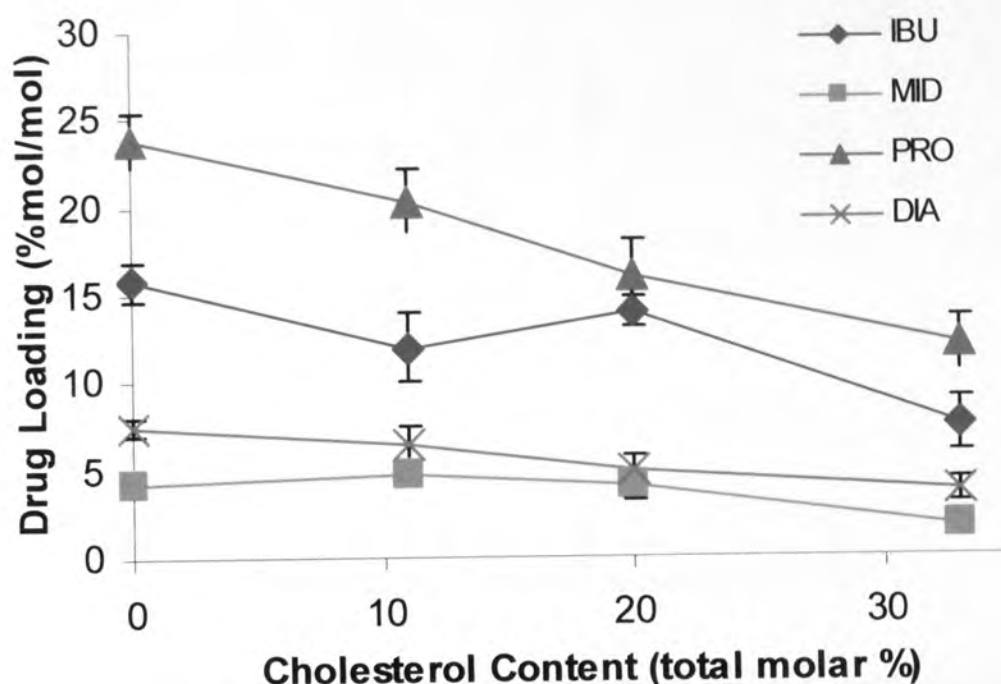


Figure 4.2 The influence of cholesterol content on entrapment. Liposomes encapsulating 1.00mg drug were formulated from PC and varying levels of cholesterol inclusion (0-33 total molar %). Drug encapsulation efficiency within MLV was determined as stated in Section 2.2.3. The drug content was analysed by UV spectroscopy at wavelength between 221-268nm. Results are expressed as the means of at least four experiments \pm S.D.

4.1.2 Liposome size and surface charge

Investigating the effect of cholesterol content on vesicle size showed that variation in cholesterol content did not generally result in any significant effect on the mean vesicle sizes (Figure 4.3). Vesicles sizes reported here generally correlated with those obtained via light microscopy (example displayed in Figure 4.4). Similarly, zeta potential did not show any relation with presence and concentration of cholesterol (Figure 4.5).

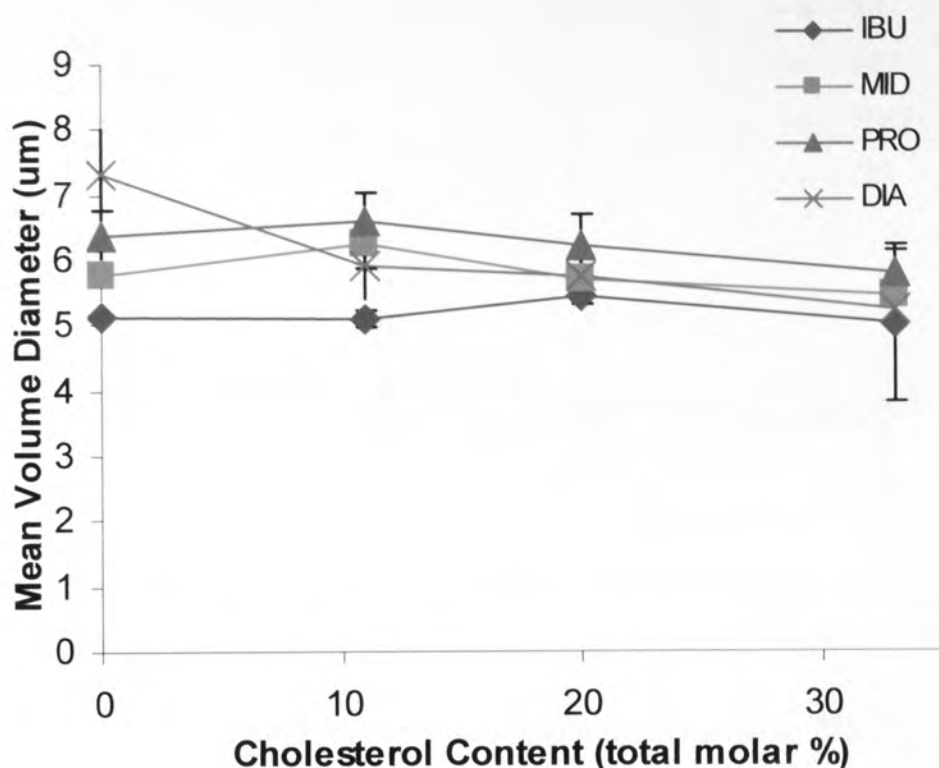


Figure 4.3 MLV mean volume distribution of liposomes encapsulating hydrophobic drugs formulated from PC and varying levels of cholesterol inclusion (0-33 total molar %) in the presence of 1.00mg of drug. Vesicle mean volume distribution was determined as described in Section 2.2.8. Results are expressed as the means of at least four experiments \pm S.D.



Figure 4.4 A light microscopy image of a diazepam-loaded PC: cholesterol (0.8: 0.2 molar ratio) liposome. Liposomes were examined at room temperature under a visible microscope (Axioskop 40) with a 40x objective lens fitted with camera at various magnifications. Images were captured using Axiovision version 3.1 software.

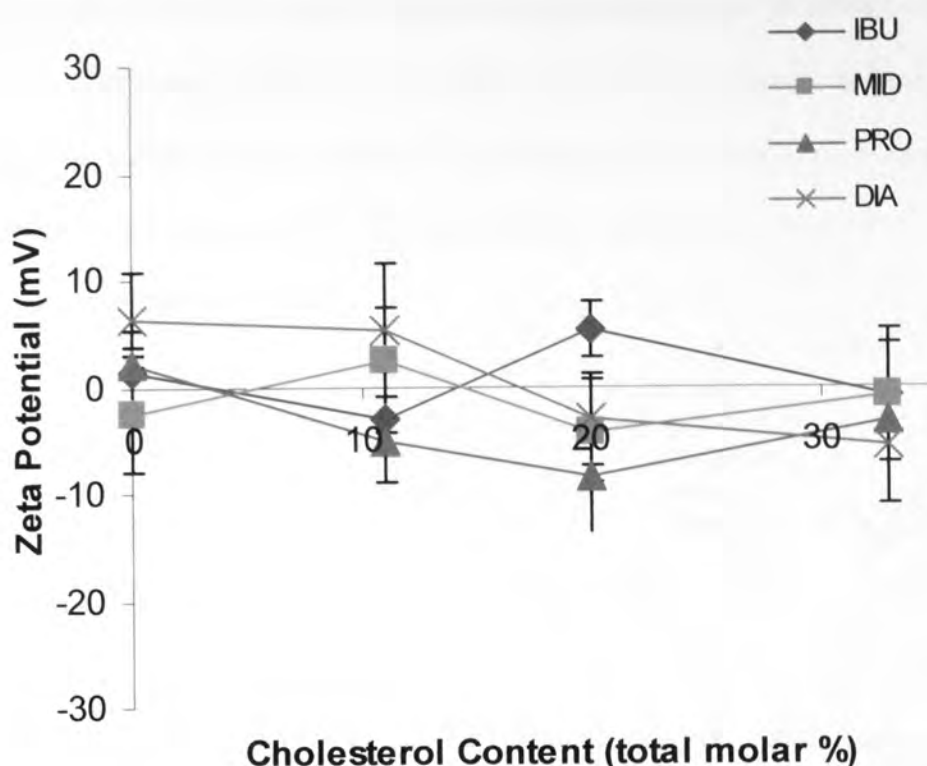


Figure 4.5 Zeta potential of liposomes encapsulating hydrophobic drugs formulated from PC and varying levels of cholesterol inclusion (0-33 total molar %) in the presence of 1.00mg. The zeta potential of vesicles was determined as described in Section 2.2.9. Results are expressed as the means of at least four experiments \pm S.D.

4.2 Vesicle formulation

Whilst a range of drugs could be incorporated in liposomes containing cholesterol, problems were encountered when attempting to optimise the cholesterol content of formulations encapsulating phenytoin and rifampicin. Effective or complete hydration of the film could not be achieved at cholesterol concentrations higher than 11 total molar % (illustration in Figure 4.6). Only a cholesterol content of 11 total molar % allowed complete hydration. This suggests that specific characteristics pertaining to the two drugs were having an adverse effect on the rigidity of the bilayer. An experiment was set-up to qualitatively investigate whether varying the concentration of phenytoin could solve the hydration issue. Five concentrations of cholesterol

ranging from 0-50 total molar % were each investigated in the presence of five drug concentrations spanning from 0-1000 μ g. PC was utilised to prepare the liposomes. A qualitative assessment was made of the efficiency of hydration of each sample with double-distilled water (pH = 5.3) and whether heating was required to achieve complete/incomplete hydration.

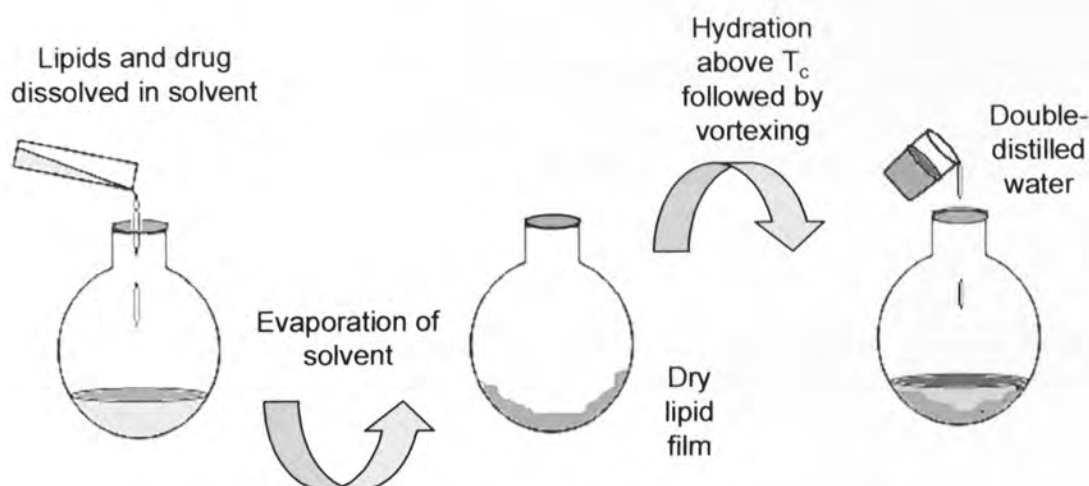


Figure 4.6 *Schematic representation of the lipid film hydration process.*

A complete hydration was noted when lipid film could visually not be seen following the addition of 2mL double-distilled water and thorough shaking. Results obtained (Table 4.1) from a qualitative assessment of the effect of drug concentration on hydration of lipid film interestingly revealed a key role of phenytoin on the effective hydration of lipid film. All vesicles prepared from 0-50 total molar % cholesterol in the absence of drug or in the presence of either 250 or 500 μ g phenytoin hydrated to form vesicles without the aid of heat. However, lipid film hydration following evaporation of all solvent appeared to become more problematic with increasing drug content. In the presence of 750 μ g phenytoin, successful hydration was attained until attempts were made to hydrate preparation containing 50 total molar % cholesterol, at which point (despite thorough shaking at 70°C), the lipid film could not completely

be removed from the glass surface of flask. Incomplete hydration was seen at lower concentrations of cholesterol when 1000 μ g of drug was included in the PC formulation. At 20% cholesterol content, complete hydration was achieved only after thorough shaking at 70°C, whereas at 33 and 50 total molar % cholesterol concentration this was not achievable even with heat induction.

Amount of phenytoin added (μ g)	Film hydrated effectively?				
	PC: cholesterol (1.00: 0.00)	PC: cholesterol (0.89: 0.11)	PC: cholesterol (0.80: 0.20)	PC: cholesterol (0.67: 0.33)	PC: cholesterol (0.50: 0.50)
0	✓	✓	✓	✓	✓
250	✓	✓	✓	✓	✓
500	✓	✓	✓	✓	✓
750	✓	✓	✓	✓	No*
1000	✓	✓	✓*	No*	No*

Table 4.1 *The effect of bilayer composition on hydration of lipid film. Liposomes were prepared from PC and various concentrations of cholesterol and phenytoin, n = 3. (* Heat applied at 70 °C)*

Interesting results seen in Table 4.1 for phenytoin prompted further investigation with another poorly water soluble model drug candidate, rifampicin. Again, the aim was to qualitatively investigate whether varying the concentration of drug affects film hydration with double-distilled water (pH = 5.3). Five concentrations of cholesterol ranging from 0-50 total molar % were each investigated in the presence of five rifampicin concentrations spanning from 0-1000 μ g using PC liposomes as the standard carrier. Heating was applied if hydration was proving difficult without thermal application.

Results (Table 4.2) acquired for hydration of lipid film incorporating rifampicin revealed conclusions similar to those observed for phenytoin. Hydration was achieved with relative ease at all rifampicin and cholesterol concentrations up to and inclusive 1000 μ g and 20 total molar %, respectively. In correlation with the outcome for

phenytoin investigation (Table 4.1), difficulties were observed when attempting to hydrate vesicles stabilised with 33 total molar % cholesterol in the presence of rifampicin concentrations higher than 500 μ g. However, unlike in the case for phenytoin, hydration difficulties were more prominent when cholesterol concentration was increased to 50 total molar % cholesterol at which point removing the lipid film from flask proved impossible using double-distilled water. This could be due to the higher Log P of rifampicin (3.72) compared to phenytoin (2.24) or its increased molecular weight (833 and 252, respectively) (Wishart *et al.*, 2006) thus making hydration of the unencapsulated rifampicin relatively more difficult.

Amount of rifampicin added (μ g)	Film hydrated effectively?				
	PC: cholesterol (1.00: 0.00)	PC: cholesterol (0.89: 0.11)	PC: cholesterol (0.80: 0.20)	PC: cholesterol (0.67: 0.33)	PC: cholesterol (0.50: 0.50)
0	✓	✓	✓	✓	✓
250	✓	✓	✓	✓	✓*
500	✓	✓	✓	✓	No*
750	✓	✓	✓	No*	No*
1000	✓	✓	✓	No*	No*

Table 4.2 The effect of bilayer composition on hydration of lipid film. Liposomes were prepared from PC and various concentrations of cholesterol and rifampicin, $n = 3$. (* Heat applied at 70 °C)

Hydration difficulties at relatively high concentrations of cholesterol could as a result of crystallisation of cholesterol when a threshold level for cholesterol incorporation into lipid bilayers is exceeded (i.e., saturation point surpassed) (Benatti *et al.*, 2007; Ohtake *et al.*, 2005). It tends to precipitate as crystals of pure cholesterol either in the monohydrate or in the anhydrous form (Loomis *et al.*, 1979; Ohtake *et al.*, 2005). The situation is made worse when a further lipophilic entity (i.e. poorly water soluble drug) is introduced competing for the same hydrophobic space within the phospholipid bilayer leading to crystals of cholesterol adsorbed to the surface of the glassware. This could explain the extreme difficulties experienced whilst attempting

to hydrate formulations consisting of 33% or 50% cholesterol and 750 or 1000µg of either drug.

4.3 The effect of liposomal inclusion of cholesterol on bilayer drug release

Biodegradability and low permeability to hydrophobic and hydrophilic substances render liposomes valuable tools for drug delivery. Therefore, a fundamental point in evaluation of liposomal drug delivery systems is to evaluate the rate at which the drug is released from the liposomal carrier systems (Yamauchi *et al.*, 2007).

Not only are *in vitro* release tests generally used in quality control of drug formulations, but they are also utilised in order to predict their behaviour *in vivo* (Nounou *et al.*, 2006). Ideally, the vesicles are desirable if they do not initiate release of drug until required (Yamouchi *et al.*, 2007). However, liposomal vehicles encapsulating drug are, by and large, unstable systems manifesting as erratic release behaviour *in vivo*- the encapsulated active compound can quite often be released into the biological fluids prior the expected target has been reached (Volodkin *et al.*, 2007). For therapeutic value, it is thus important that drugs are retained in liposomes for an appropriate time (Yamauchi *et al.*, 2007). Among the various factors that dictate the liposomal release of drug, the bilayer composition is an important factor (Devaraj *et al.*, 2002; Matsuki *et al.*, 2007).

The effect of cholesterol concentration on the release of a hydrophobic drug was examined by preparing liposomes containing three concentrations (11, 20 and 33 total molar %) of cholesterol and compared to formulation containing no cholesterol. Propofol was selected as the model drug candidate since drug incorporation was

highest with propofol when compared to other drugs considered (Section 4.1.1). Propofol release from the four formulations was studied in physiological buffer, pH = 7.4, at a constant temperature of 37°C.

Percent propofol released versus time plot is shown in Figure 4.7a. Generally, significant ($P < 0.05$) changes in release were observed with change in cholesterol content in the bilayer of liposome. The drug release profile of formulation absence of cholesterol was characterised by an initial burst effect (more than 40% drug released in first 24hour). A rapid release decreased considerably when formulation was supplemented with cholesterol (11 total molar %). An increase in cholesterol to 20% yielded a similar release profile. After 72hour, about 50% of encapsulated propofol had undergone leakage compared to about 80% for sample lacking in cholesterol. A further increase in the compound (33 total molar %) resulted in a significant ($P < 0.05$) slow-down in the release rate, with only 40% of loaded-drug measured following 72hour incubation at 37°C. The release rates appeared to show some relation with encapsulation. Results displayed in Section 4.1.1 state a gradual reduction in propofol encapsulation with an increase in concentration of cholesterol, thus suggesting a permeability-reducing effect by the substance. This theory appears to be supported by the propofol-release results obtained here, where a general reduction in permeability was observed with decreasing levels of cholesterol.

The release profiles of liposomes (Figure 4.7a) show that presence of cholesterol in the bilayer stabilises and reduces their permeability to the hydrophobic propofol up to 72hour. Similar conclusions have been reported in literature. Recently, release profiles cited by Hathout *et al.* (2007), demonstrated that the increase in the molar

ratio of cholesterol in the prepared liposomal formulations progressively decreased the release of acetazolamide from the vesicles. Although Gopinath *et al.* (2004) did not show any consistent relation between release rate of another drug azidothymidine and cholesterol, they revealed a significant reduction in permeability when the content of cholesterol was enhanced from 18% to 45%, therefore indicating a more stable formulation in the presence of the higher quantity of the substance.

The higher membrane permeability of the liposomes without cholesterol compared to the version with the compound could be due to the presence of defects in the former, more fluid, membrane induced by the absence of cholesterol (Volodkin *et al.*, 2007). The presence of cholesterol and saturated phospholipids is understood to be key factors for reducing membrane permeability of amphipathic drugs (Drummond *et al.*, 1999). This well-recognised ability of cholesterol to induce membrane-stability is thought to occur via an interaction between the rigid hydrophobic ring structure of the molecule and the alkyl side-chains of phospholipids, which ultimately decreases membrane fluidity (Wiseman *et al.*, 1993). Cholesterol modifies the fluidity by increasing the orientation order of the relatively mobile hydrocarbon chains of liquid-crystalline phospholipid bilayers decreasing bilayer permeability, and reducing the efflux of the entrapped drug, resulting in prolonged drug retention and, when present in sufficient quantity, it is understood to abolish the gel-to-liquid phase transition endotherm of bilayers (Hathout *et al.*, 2007; Ohtake *et al.*, 2005; Vilcheze *et al.*, 1996).

Surface pressure measurements (Rogerson *et al.*, 1987) on monolayers of nonionic surfactant/cholesterol mixtures have revealed a compressing influence of cholesterol

as manifested by the reduction in the effective area per molecule as the presence of the stabiliser in the monolayer is increased. This was attributed to the surfactant monolayers accommodating cholesterol in their molecular cavities as they formed vesicles. This space-filling mechanism of the molecules is accepted to be accountable for the observed reduced permeability of cholesterol-harboured membranes compared to cholesterol-absent membranes (Devaraj *et al.*, 2002).

4.3.1 Release Kinetics

Three of the most common kinetic profiles are zero-order, first-order and Higuchi (Hughes, 2005). To determine the mechanism of drug release from the four formulations, the release data (Figure 4.7a) was treated according to zero-order (cumulative amount of drug released vs. time), first-order (log cumulative percentage of drug released vs. time) and Higuchi's (cumulative percentage of drug released vs. square root of time) models (Khatun *et al.*, 2004) with results presented in Figures 4.7b, c and d, respectively.

Results obtained (Figure 4.7) for release of propofol from PC-only bilayer were found to follow zero-order release kinetics (Table 4.3). The release mechanism changed with formulation; cholesterol content appeared to be predominant controlling factor. The presence of cholesterol appeared to transform the kinetics from a zero-order for formulation absent of the stabiliser to a first-order release thus indicating the release mechanism to be changeable, a process reported in literature (Xu and Sunada, 1995). As the cholesterol content increased, cumulative drug release rate generally decreased, and the release mechanism remained a first-order profile (Table 4.3).

Looking closer into the kinetics of propofol release from the cholesterol-containing liposomes, the release data from time zero to 72hour were found to best fit first-order model based on the magnitude of the correlation coefficient obtained for zero-order, first-order and Higuchi's diffusion model (Figures 4.7b, c and d, respectively); when the data were plotted according to the first-order model, the formulations showed a good linearity (Figure 4.7c), with R^2 values between 0.956 to 0.959 (Table 4.3). Whilst in comparison, formulation absent of cholesterol revealed a zero-order release profile (constant rate of drug release from device) possibly reflecting its relatively porous, more fluidised membrane structure compared with its more rigid and theoretically less permeable cholesterol-containing counterparts. Interestingly, low correlation coefficients were obtained for the Higuchi model (Table 4.3), indicating that neither of the formulations followed a diffusion controlled release model reported for various hydrophobic drug-loaded liposomal preparations in literature (acetazolamide in Hathout *et al.*, 2007; dibucaine in Nounou *et al.*, 2006). This was a surprising finding as encapsulation figures for the four formulations prior to release studies suggested the vehicles may follow a Higuchi's model as the solubility of propofol loaded in the drug-delivery device was above its solubility (153 μ g/mL; Altomare *et al.*, 2003), a fundamental requirement in order for a given device to exhibit a diffusion-controlled release mechanism (Lee *et al.*, 1998).

Formulation	R^2		
	Zero order plot	First order plot	Higuchi's plot
PC: cholesterol (1.00: 0.00)	0.9806	0.9393	0.9504
PC: cholesterol (0.89: 0.11)	0.9119	0.9593	0.8091
PC: cholesterol (0.80: 0.20)	0.9137	0.9582	0.8250
PC: cholesterol (0.67: 0.33)	0.9140	0.9563	0.8255

Table 4.3 Kinetic values of release of propofol from different liposomal formulations using the correlation coefficient parameter (R^2).

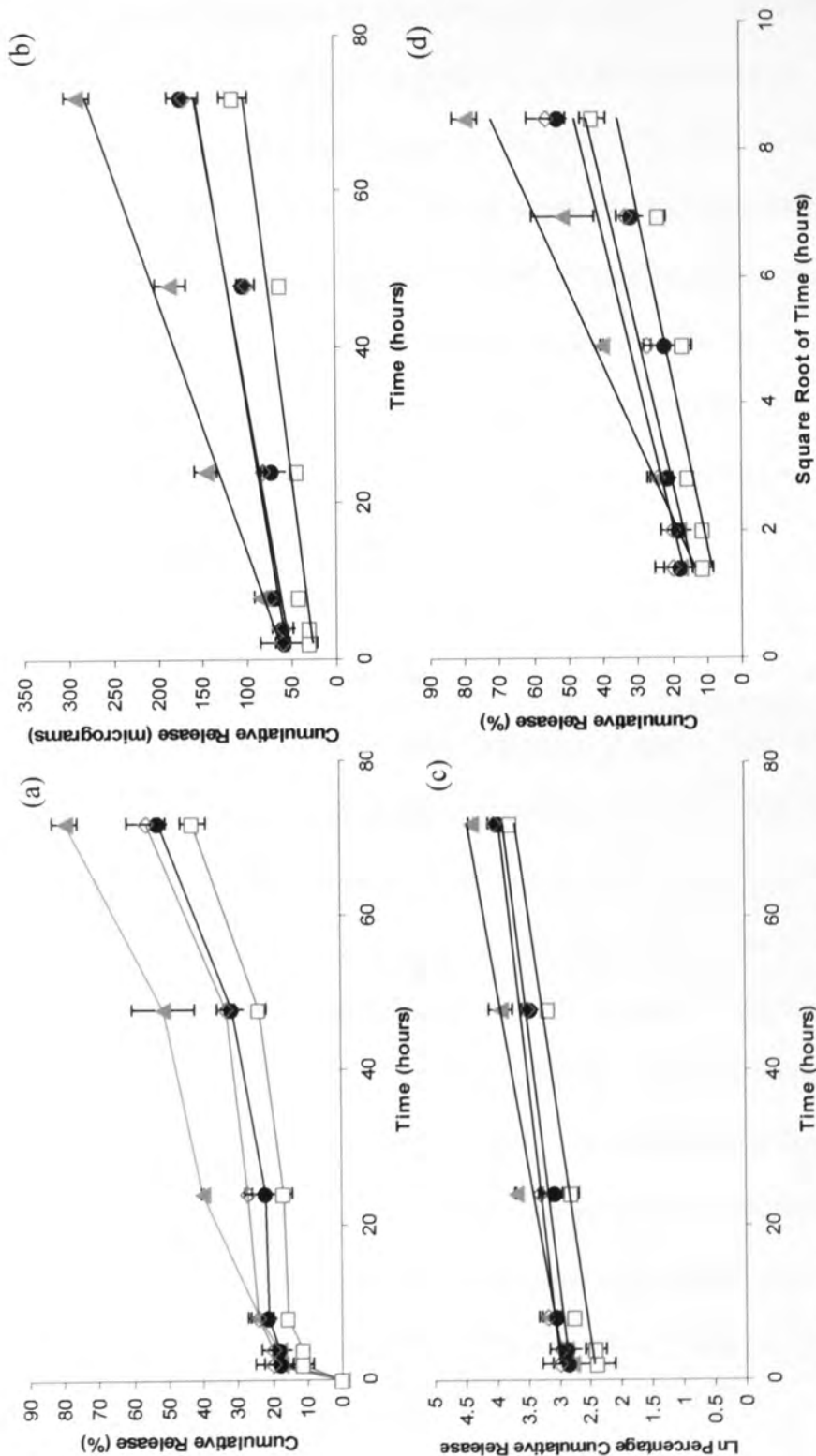


Figure 4.7 Effect of cholesterol concentration on the release of propofol from liposomes. The cumulative release profile of propofol under physiological conditions from Chol-0% (▲) Chol-11% (●), Chol-20% (◊), Chol-33% (□), formulations in aqueous buffer, pH = 7.4, at 37°C (a). The data are replotted according to zero-order (b), first-order (c) and Higuchi's (d) models. Propofol was encapsulated and drug release was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27,200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments \pm S.D.

4.4 Interactions between DSPC and cholesterol in monolayers at the air/water interface

Langmuir monolayers are an excellent model system to investigate two-dimensional ordering with temperature and pressure, two thermodynamical variables, directly capable of being controlled (Castelli *et al.*, 2007; Kaganer *et al.*, 1999). The method can be exploited as an uncomplicated model for the lipid-water interface of cell membranes in order to probe processes at surfaces of membranes (Cardenas *et al.*, 2005), yielding phase diagrams of phospholipids in the form of surface pressure/mean molecular area (Π -A) isotherm curves (Castelli *et al.*, 2007). The method has undergone a revolution within the last decade evidenced by an extensive array of literature (Kaganer *et al.*, 1999).

It is an extremely sensitive technique with minor singularities of the isotherm potentially implying structural phase transitions (Kaganer *et al.*, 1999). The technique can be used to thermodynamically analyse interactions between components in mixed monolayers enabling the investigator to draw conclusions with respect to both the level of affinity between two components and their molecular arrangement at the air/water interface (Hac-Wydro *et al.*, 2007). For example, studies by Hac-Wydro and Wydro (2007) found that a saturated fatty acid (i.e. stearic acid) rendered phospholipid membranes more rigid, while the inclusion of an unsaturated fatty acid (i.e. oleic acid) increased its fluidity. Furthermore, an increasing quantity of the saturated fatty acid gradually destabilised the model membrane whereas in comparison, a small proportion of its unsaturated analogue made the membrane thermodynamically more stable.

Experiments using Langmuir monolayers could also offer a degree of flexibility. For instance, a component could be introduced into the sub-phase of the system allowing assessment of interactions with a surface component: Souza *et al.* (2004) examined the interaction of diclofenac with PC monolayers via the introduction of the former into the subphase with its effects being characterised with surface pressure measurements. Interestingly, they also studied the effect of encapsulating diclofenac into bilayers of fully-formed PC liposomes by injecting the vesicles into the aqueous subphase and probing the kinetics of lipid film formation at a constant area following the disruption of the liposomal structure.

For a better understanding of the interactions and complex formation between phospholipids and cholesterol, a systematic investigation was undertaken on the monolayer properties yielded by various concentrations of cholesterol and DSPC (DSPC was used in place of PC as a pure sample can be obtained from using the former; PC is a mixture of compounds). Additionally, since this study has been performed before (Dynarowicz-Latka and Hac-Wydro, 2004), it was used as a standard whilst attempting to optimise the technique for further investigations.

The experiments were based on the aforementioned Langmuir monolayer technique- phospholipids spread over an aqueous subphase, offering monomolecular distribution and the resulting variation of the available area per molecule on the surface (Castelli *et al.*, 2007). The compression modulus-area and mean molecular area parameters were used to study the interactions between DSPC and cholesterol. Mixtures (and the one-component films) of the phospholipid and the stabiliser at molar fractions ranging from 0.125 to 0.5 were spread at the air/water interface (pH = 5.5).

4.4.1 DSPC monolayers

At low pressures ($\sim 1\text{mN/m}$) the Π -A isotherm (Figure 4.8) for a pure DSPC monolayer at the air-water interface was in the gaseous state. The liquid-expanded phase was observed between 1 and 4mN/m and the liquid-condensed found above 4mN/m . The monolayer attained solid state at approximately 40mN/m . The average measured cross-sectional area of the DSPC molecule was $51.0\text{\AA}^2/\text{molecule}$. The Π -A isotherm for pure DSPC observed here is in agreement with results collated by Cardenas *et al.* (2005) and Hac-Wydro *et al.* (2007).

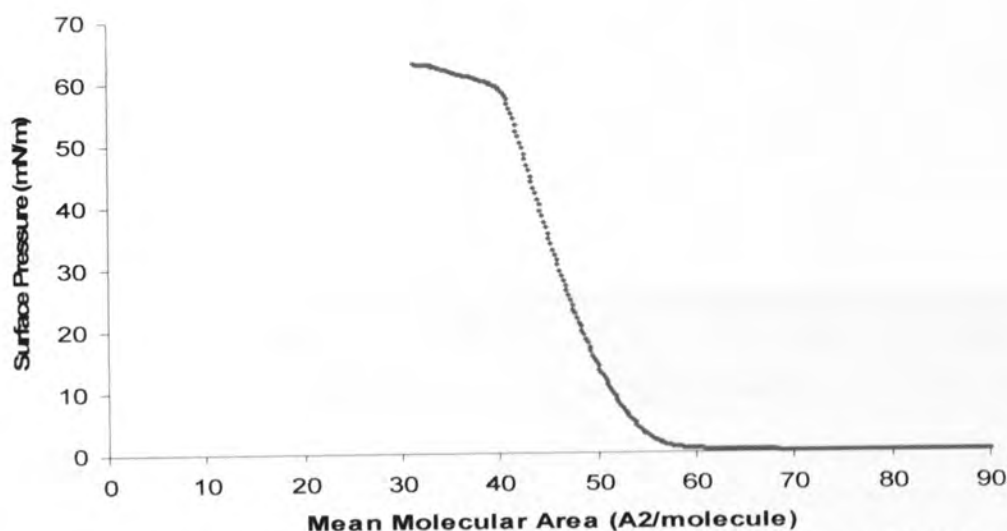


Figure 4.8 The surface pressure-area isotherm of pure monolayer of DSPC at the air/water interface (at 20°C). Results are expressed as the means of three experiments.

4.4.2 Cholesterol monolayers

In the case of cholesterol, a swift, practically linear, increase of surface pressure up to point of collapse (at approximately 42mN/m , Figure 4.9) indicates a closely-packed monolayer structure that exists as a solid-phase during the compression with mean molecular area avoiding radical alterations during the process, in relation with the flat ring and rigid configuration of cholesterol (Barnes and Gentle, 2005). The phase characteristics and collapse parameters, obtained from the isotherm, are in good

correlation with data obtained by Korchowiec *et al.* (2006) and Zhao and Feng (2006) where similar conclusions were published. In literature, the extrapolated molecular area of cholesterol has been measured to be 38.3 and 40.0 Å²/molecule (Zhao and Feng, 2006 and Minones Jr. *et al.*, 2006, respectively), which are approximately equivalent to the extrapolated area of the monolayer (39.5 Å²/molecule) determined here for cholesterol.

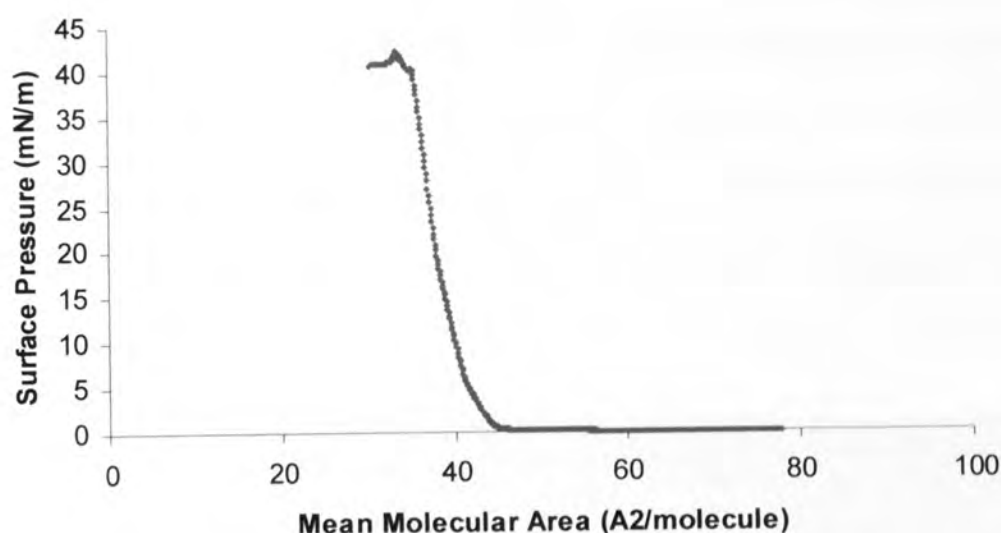


Figure 4.9 The surface pressure-area isotherm of pure monolayer of cholesterol at the air/water interface (at 20°C). Results are expressed as the means of three experiments.

4.4.3 Monolayers of mixtures

To investigate the intermolecular packing of phospholipids, the monolayers formed from mixtures of DSPC and cholesterol at ratios of (0.89: 0.11, 0.80: 0.20 and 0.67: 0.33 molar ratios) were also measured (Figure 4.10). All mixed isotherms lie in-between those for pure components. The isotherms for mixtures of DSPC with cholesterol shift systematically towards the curve for the one-component monolayer of the latter. The obtained mean molecular areas for mixed solutions shown in Table 4.4 were smaller than the anticipated ones for the ideal mixtures (negatively deviating

by 8.9 and 11.4% for mixtures containing 11 and 33 total molar % cholesterol, respectively). The cholesterol appears to induce a 'condensing effect' in mixed phospholipids/cholesterol monolayers at the air-water interface (as suggested in Dynarowicz-Latka and Hac-Wydro, 2004, Korchowiec *et al.*, 2006 and Minones Jr. *et al.*, 2006), possibly as a result of good bilayer interpacking, thereby indicating a strong interaction between the two components.

The insertion of cholesterol into the flexible DSPC bilayers of the liposomes is said to modify their phase behaviour- creating order, enhancing the packing density and therefore an increased chain rigidity- resulting in reduced permeability (Gregoriadis, 1985; Hadgraft *et al.*, 1996; Volodkin *et al.*, 2007). This could explain the reduced encapsulation and cumulative release of drug with increase in cholesterol concentration observed earlier (Section 4.1.1 and Section 4.3.1, respectively).

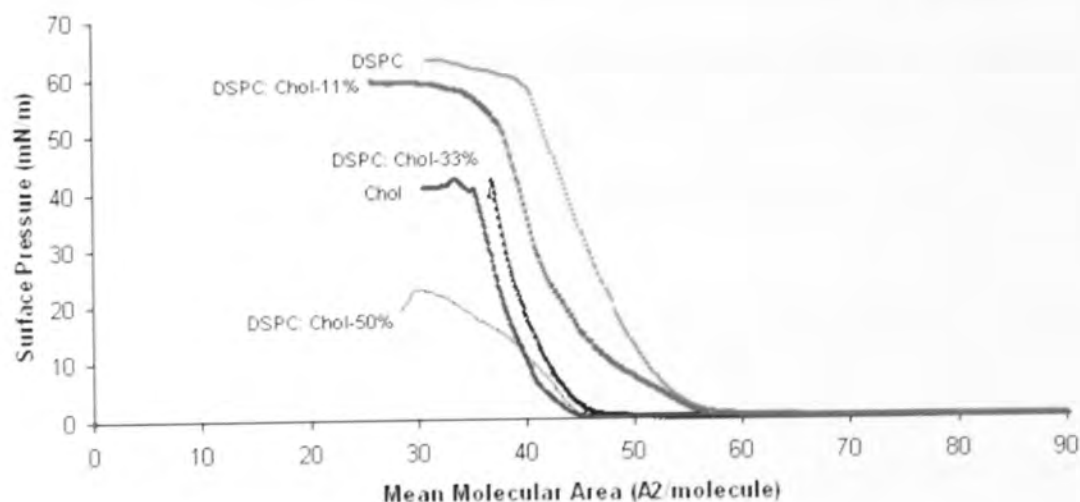


Figure 4.10 The surface pressure-area isotherms of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC and cholesterol. Results are expressed as the means of three experiments.

Formulation	Extrapolated area at zero pressure ($\text{\AA}^2/\text{molecule}$)	Ideal extrapolated area at zero pressure ($\text{\AA}^2/\text{molecule}$)	Deviation from ideality (%)	Collapse pressure (mN/m)
DSPC (pure)	51.0	-	-	63.2
Cholesterol (pure)	39.5	-	-	44.2
DSPC: cholesterol -11%	45.3	49.7	-8.9	59.3
DSPC: cholesterol - 33%	41.8	47.2	-11.4	42.5
DSPC: cholesterol - 50%	45.0	44.5	+1.1	23.2

Table 4.4 *The experimental and ideal extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC and cholesterol. Results are expressed as the means of three experiments.*

4.5 Conclusions

It is apparent from studies that cholesterol influences the encapsulation efficiency and release of hydrophobic drugs and thus optimisation is essential. Furthermore, it was also demonstrated that certain drugs pose problems during the hydration stage of synthesis when present with relatively high concentrations of the stabiliser, possibly as a result of competition between the two entities for hydrophobic packing space at bilayer level. Since the same lipid components were utilised for the preparation of liposomes, the zeta potential values were quite similar for all preparations (approximately neutral). Moreover, there was no statistically significant difference between average volume diameters of the liposomal formulations measured by laser diffraction or via estimation by electron microscopy image analysis.

It was also shown that the release of propofol from PC liposomes is dependent on the concentration of cholesterol and can therefore be controlled by selecting the appropriate combinations of these two components. Different kinetic models (zero-order, first-order, and Higuchi's equations) were applied to interpret the drug release kinetics from the liposomal systems. The best linear relationship appeared to be a zero-order kinetic model for PC-only formulation. But when cholesterol was present, a straight line with relatively high level of linearity was observed for a first-order

model, thus indicating the release mechanism to be changeable, a process reported in literature (Xu and Sunada, 1995).

Langmuir plots of mixed DSPC and cholesterol monolayers revealed significant deviations from ideality, proving the existence of strong interactions between the two components at the bilayer level possibly explaining the marked difference in encapsulation and release of poorly water-soluble drug with increase in cholesterol content.

Since past reports suggest that the presence of cholesterol provides a degree of physical bilayer stability, and that, regardless of bilayer charge, liposomes rich in cholesterol were more stable than cholesterol- poor/cholesterol- free liposomes (Zhang *et al.*, 2005), a cholesterol content of 11 total molar % was implemented for all further formulations.

Chapter Five:

The role of liposome bilayer composition on drug solubilisation

5.0 Introduction

The low solubility of drugs like amphoterecin B in clinically relevant aqueous formats is a problem not easily addressed with many drugs (Lincopan *et al.*, 2006). The application of liposomes to enhance the delivery of drugs has been investigated extensively, but only recently has interest in these systems turned to exploring their potential as solubilisation agents. The delivery of drugs by lipid vesicles to treat a wide range of diseases entails a comprehensive examination of the physico-chemical features in order to forecast their *in vivo* behaviour and stability (Bermudez *et al.*, 1999). For example, for the therapeutic treatment of mycobacterium tuberculosis, the composition of liposomal bilayer is critical for targeting liposomes to the lung and for attaining stable formulations (Bermudez *et al.*, 1999). Drug-delivery and biological systems such as vaccines and cells must often be stored for extended periods of time; therefore, attaining long-term stability in these systems has been a long-standing goal of the pharmaceutical and biomedical industries (Ohtake *et al.*, 2005). Typical examples of markers for stability include the ability of a liposomal preparation to retain encapsulated solutes (Kirby and Gregoriadis, 1999) and changes in vesicle dimensions over time (Terzano *et al.*, 2005).

The relative significance of the structural characteristics of the membrane (i.e. alkyl chain length of lipid, geometry, nature and charge of the head-group and presence of stabiliser) must be established in order to portray the mode of action of poorly water-soluble drugs on membranes at the molecular level (Faucon *et al.*, 1995), on the basis of certain markers (i.e. encapsulation, stability and release) evidenced on model systems. It is

recognised that vesicular size, surface properties, and overall stability substantially influence liposomal efficiency (Fatouros and Antimisiaris, 2002).

The aims here were to prepare and characterise various liposome-based formulations to understand the factors responsible for the potential development of an efficient and stable liposomal formulation and finally to evaluate any potential toxicity concerns of the liposome-based formulations. Therefore, this work investigated the role of alkyl chain length, geometry and a near-fully charged bilayer on the encapsulation of poorly water-soluble model drug candidates. Short-term (28day) stability studies were performed to investigate drug loss from liposome formulations under different storage conditions. The morphology of the various formulated vesicles was observed via transmission electron microscopy and optical microscopy. As toxicity remains a key obstacle in the development of pharmaceutical formulations, *in vitro*-based studies were performed scrutinising the relationship between toxicity and hydrophobic chain length and geometry as well as lipid head-group charge.

Since in the case of drug solubilisation, the drug is loaded within the liposomal membrane rather than the aqueous core, it was interesting to examine the influence structural features of drugs have on their encapsulation into bilayers to gain insight to any structural parameters which dictate drug loading and retention within the bilayer structures.

5.1 Studies of liposomes prepared from phosphatidylcholine and its derivatives

Considering the results in Section 4.1.1, the inclusion of cholesterol as stabiliser at 11 total molar % revealed enhanced drug encapsulation features hence this quantity was employed for all formulations examined in this section.

5.1.1 The influence of lipid alkyl chain length on liposomal encapsulation of poorly water-soluble drugs

The effect of lipid alkyl chain length on incorporation of model drug candidates (diazepam, midazolam or propofol) was investigated using liposomes prepared from PC (C_{12} average alkyl chain length) and its derivatives, DMPC (C_{14} alkyl chain length), DPPC (C_{16} alkyl chain length), DSPC (C_{18} alkyl chain length) and C_{24} PC (C_{24} alkyl chain length). Each formulation was prepared with 11 total molar % cholesterol concentration.

Results in Figure 5.1 revealed an increase in propofol and midazolam bilayer-encapsulation with an increase in lipid chain length in the order of C_{24} PC > DSPC > DMPC \geq DPPC = PC (Figure 5.1a) and DSPC \geq DPPC > DMPC > PC (Figure 5.1b), respectively ($P < 0.05$). This correlates with previous work investigating the solubilisation of ibuprofen in liposomes (Mohammed *et al.*, 2004) where they revealed a trend of increasing encapsulation levels of the water-insoluble ibuprofen with increasing lipid chain length in the order of C_{24} PC > DSPC > DMPC > PC, with drug loading being improved by approximately 50% when C_{24} PC was substituted for PC. Furthermore, Gregoriadis (1973) showed that the longer alkyl chain DPPC was superior to PC in enhancing encapsulation of actinomycin D, a slightly water-soluble antibiotic. This trend does not appear to be exclusive to liposomes: Ismail *et al.* (1970) established improved

solubilities of certain barbiturates in micelles of polysorbates of increasing hydrophobic chain length. This trend of increasing drug loading with increase in alkyl chain length may be due to the increased bilayer lipophilic area within systems formed by these longer lipid bilayers for the hydrophobic drug to accommodate within (Figure 5.2) (Mohammed *et al.*, 2004). This theory is further backed by a recently published literature (Dan, 2007) which states that bilayer thickness increases with increasing alkyl chain length of phospholipid.

However interestingly, the trends obtained for propofol and midazolam did not appear to parallel with the results obtained for diazepam incorporation by the PC derivative-based liposomes, where the reverse was demonstrated (increasing diazepam incorporation with decreasing lipid alkyl chain length in the order of $PC > DMPC \geq DPPC = DSPC \geq C_{24}PC$) (Figure 5.1c). This suggests that the increasing chain length is creating a less favourable environment for the hydrophobic drug to incorporate within the phospholipid bilayer. This could be attributed to the significantly lower lipophilicity of diazepam ($\text{Log } P = 2.99$ contrasted with $\text{Log } P = 3.87$ and $\text{Log } P = 3.84$ for midazolam and propofol, respectively) (Wishart *et al.*, 2006).

Substituting a derivative for PC did not appear to yield a trend in the mean vesicle sizes or zeta potential (Table 5.1).

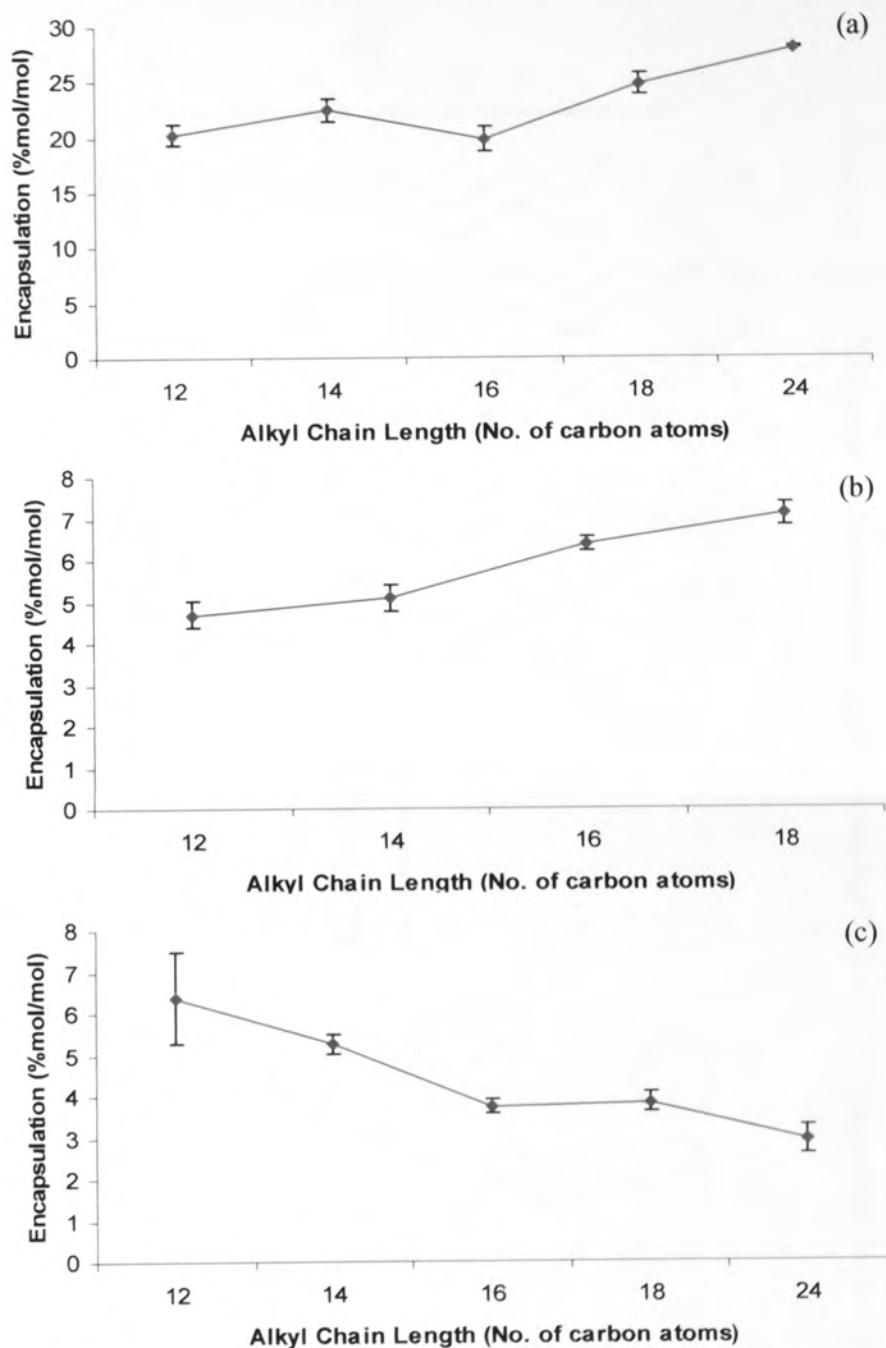


Figure 5.1 The influence of alkyl chain-length on encapsulation of propofol (a), midazolam (b) and diazepam (c). The drug loading of vesicles was determined as stated in Section 2.2.3. The drug content was analysed by UV spectroscopy at 228nm (diazepam), 258nm (midazolam) or 268nm (propofol) wavelengths. Results are expressed as the means of four experiments \pm S.D.

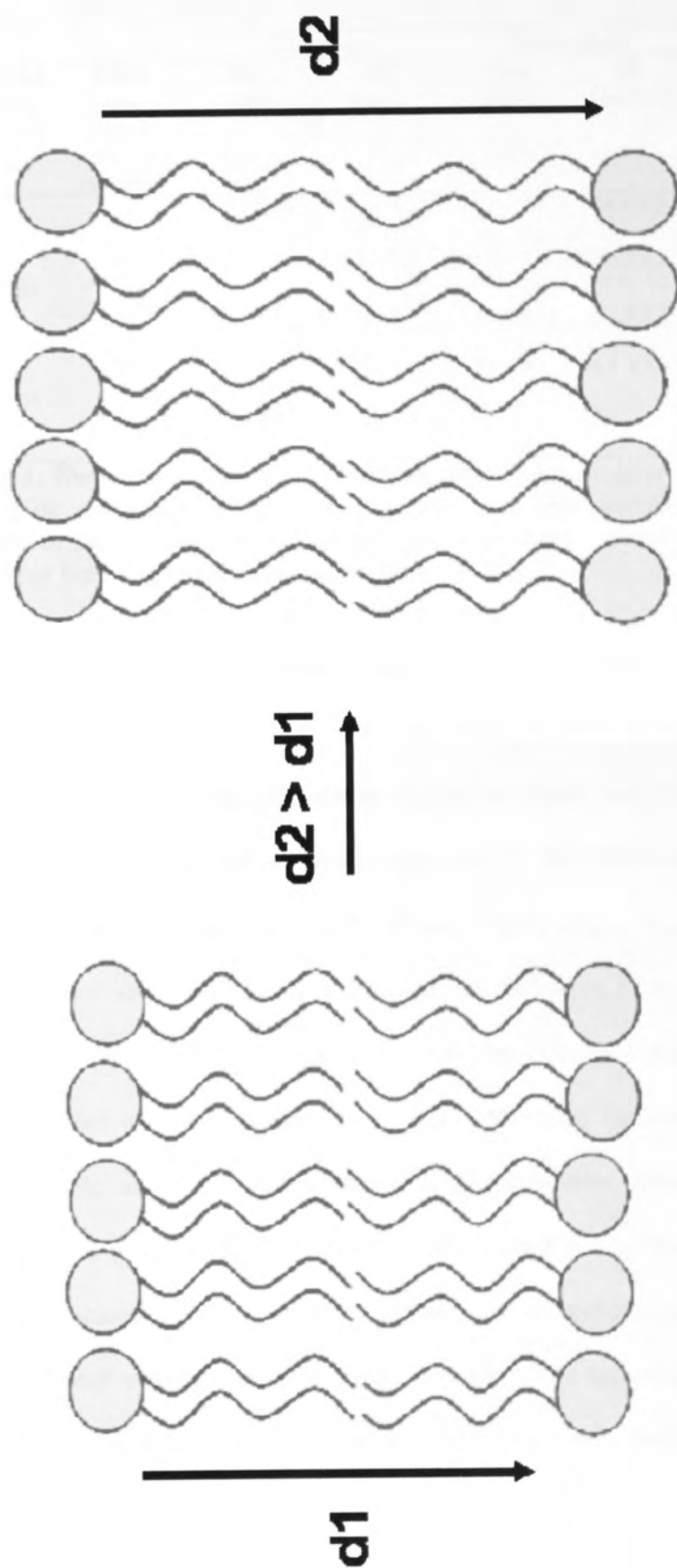


Figure 5.2 Diagram proposing the possible increase of hydrophobic region with increasing lipid tail length. (d = diameter)

Formulation (0.89: 0.11 molar ratio)	Lipid tail length (no. of carbons)	Diazepam		Midazolam		Propofol	
		Size (μm)	ZP (mV)	Size (μm)	ZP (mV)	Size (μm)	ZP (mV)
PC: cholesterol	12	5.9 ± 0.5	5.4 ± 6.2	6.2 ± 0.6	2.4 ± 5.0	6.6 ± 0.5	-5.1 ± 0.9
DMPC: cholesterol	14	9.0 ± 1.3	3.2 ± 2.2	8.2 ± 0.6	-0.3 ± 6.2	7.8 ± 0.9	-2.6 ± 0.5
DPPC: cholesterol	16	9.6 ± 0.3	5.4 ± 6.0	7.5 ± 0.1	-4.1 ± 3.2	8.7 ± 1.0	-3.6 ± 1.5
DSPC: cholesterol	18	7.8 ± 1.7	-3.9 ± 3.9	8.4 ± 0.2	-1.9 ± 3.6	8.5 ± 1.1	-4.4 ± 3.7
C ₂₄ PC: cholesterol	24	6.3 ± 1.9	-5.4 ± 6.5	-	-	7.5 ± 0.78	-1.8 ± 4.3

Table 5.1 The influence of alkyl chain length of phospholipid on liposome size and charge. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. Results are expressed as the means of at least four independent experiments \pm S.D.

5.1.2 Temperature effects on encapsulation

As stated in Section 2.2.1, the lipid film used in the preparation of liposomes is hydrated above the liquid-crystalline temperature (T_c) of the lipids with 2mL of double distilled water, with preparations left to stand above the T_c for 30min during which time the liposomes are formed. Thus in case for DSPC, a lipid with a T_c of 55°C, the hydration media is heated above its T_c to help facilitate the formation of the vesicles. Heating can also improve drug solubility in aqueous conditions (Florence and Attwood, 1998). For example, earlier work by Bustamante *et al.* (1998) tested the solubility of paracetamol and acetanilide at several temperatures in dioxane-water mixtures relative to their thermodynamic magnitudes. DSC analysis was carried out on the original powders and on the solid phases following equilibration with the solvent mixture. The drugs revealed linear behaviour at the temperature range examined. The apparent enthalpies of solution were endothermic passing a maximum at 50% dioxane with two different mechanisms,

entropy and enthalpy, suggested to be the driving forces that enhanced the solubility of both drugs.

It may be possible that the heating of the hydration media itself is the driving force behind the increase in bilayer encapsulation with increasing alkyl chain length (Figure 5.1) (and therefore increase in the application of heat, accordingly) as opposed to the greater availability of hydrophobic region. To examine the possibility that heating up the solution of liposomes entrapping drug could influence solubility and/or drug incorporation, PC and DSPC liposomes encapsulating diazepam were prepared; the temperature of hydration media for one set was maintained at ambient temperature whereas the other was hydrated at 70°C. Results generated revealed no significant variation in drug loading (Table 5.2) therefore ruling out the possibility that temperature employed in the preparation of liposomes influenced drug loading within the liposome formulations.

Formulation (0.89: 0.11 molar ratio)	Encapsulation (%)	
	Just above respective T _c	At 70 °C
PC: cholesterol	32.6 ± 5.7	29.3 ± 4.3
DSPC: cholesterol	19.7 ± 1.1	17.9 ± 3.6

Table 5.2 *Liposomal encapsulation of diazepam at two different temperatures. UV analysis carried out at 228nm. Results are expressed as the means of three experiments ± S.D.*

5.1.3 The influence of lipid alkyl chain length on drug release

To investigate the role of the alkyl chain length on the drug release rate offered during *in vivo* temperature, propofol-encapsulated liposomes prepared from PC, DMPC, DPPC and

DSPC (stabilised with addition of 11 total molar % cholesterol) at 37°C were investigated.

A general increase in cumulative release rate at 72hour with decreasing lipid chain length was observed for subjects stabilised with cholesterol in the order of PC > DMPC = DPPC > DSPC at 37°C (data at 72hour, $P < 0.05$) (Figure 5.3). Generally, propofol release followed a common release profile for all four formulations. Preparation containing DSPC released the least amount of drug whereas PC-cholesterol sample displayed highest quantity of leakage during analysis at 72hour time-point. Cumulative release rate for DPPC- cholesterol and DMPC- cholesterol were indistinguishable throughout the three-day study (Figure 5.3).

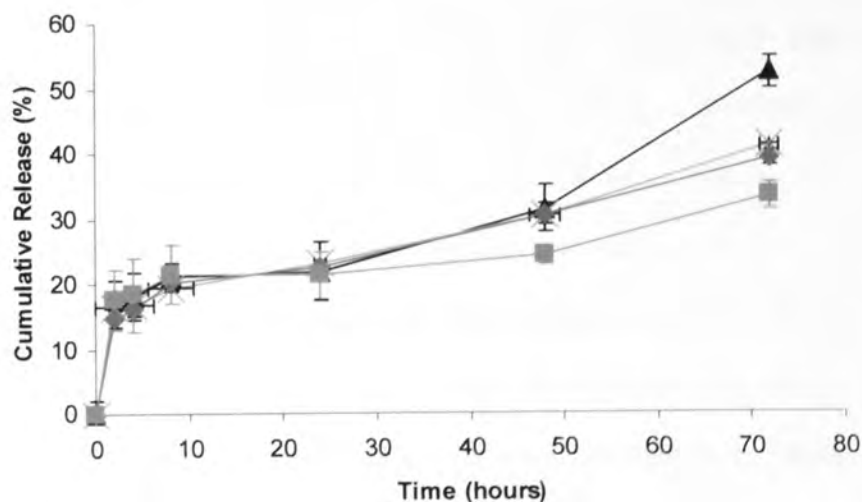


Figure 5.3 The effect of phospholipid alkyl chain length on drug release. Propofol release under physiological conditions from PC- cholesterol (▲) DMPC- cholesterol (×), DPPC- cholesterol (◆), DSPC- cholesterol (■), formulations in aqueous buffer, pH = 7.4, at 37°C. Propofol was encapsulated and drug release was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27,200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments \pm S.D.

The influence of alkyl chain length on release have previously been detailed. Taylor *et al.* (1990) reported that liposomes formulated from the mixture of DMPC with equimolar portions of cholesterol displayed an enhanced leakage than those prepared from the longer chain DPPC-cholesterol mixtures. Bilayer drug release is recognised to be influenced by the phase transition temperature (T_C) of the lipid excipients within the vesicles (Mohammed *et al.*, 2004). The phase transition temperature of liposomes is significantly influenced by the alkyl hydrocarbon chain length (Yoshioka *et al.*, 1994); as the hydrocarbon chain length is increased, Van der Waal's interactions between the alkyl chains become stronger necessitating additional energy to disrupt the ordered packing, thus increasing phase transition temperature. Gel phase bilayers are therefore less permeable than the corresponding fluid bilayers (Liu *et al.*, 2001). As a consequence, at 37°C, both PC ($T_C < 0^\circ\text{C}$) and DMPC ($T_C = 23^\circ\text{C}$) vesicles will be in the fluid state leading to an enhanced drug-leakage when weighed against their higher transition temperature counterparts, DPPC ($T_C = 41^\circ\text{C}$) and DSPC ($T_C = 55^\circ\text{C}$) (Mohammed *et al.*, 2004; Saarinen-Savolainen *et al.*, 1997). Similarly, conclusions obtained by Volodkin *et al.* (2007) state that bilayer permeability depends substantially on the lipid phase state with liposomes (DPPC) in the solid state retained the encapsulated carboxyfluorescein dye for a long time whereas continuous release was registered for "fluid" vesicles (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine). Furthermore, remote-loading of drug just prior to injection is reported to be required for compositions containing more "fluid" components, i.e. eggPC lipid, as a result of an elevated level of leakage during storage (Drummond *et al.*, 1999).

5.1.4 The effect of phospholipid alkyl chain length on stability

The influence of phospholipid alkyl chain length on the physical stability of liposomes during a 28-day storage period at 4°C and 25°C was examined. Stability can refer to various different features of a liposomal formulation carrying a drug: chemical stability of both drug and lipid constituents, colloidal stability, and drug retention (Drummond *et al.*, 1999). Liposomal stability upon storage is defined as the degree a formulation retains both its original mean volume distribution and its drug load. Instability occurs when, for example, the vesicle size distribution spontaneously increases due to fusion of colliding vesicles (Gregoriadis, 1985). Therefore, for successful commercial utilisation of liposomes it is imperative that adequate substance can be incorporated for the projected application and further that it is retained until their subsequent use (Drummond *et al.*, 1999; Ishida *et al.*, 2002; Kirby and Gregoriadis, 1999).

The mean volume diameter is a further marker of stability of the vesicles (du Plessis *et al.*, 1996). Radical changes in the vesicular mean size and sometimes, vesicle shape can result from aggregation, flocculation and/or coalescence as well as osmotic shrinkage or swelling (depending on the osmotic gradient) (Uchegbu and Florence, 1995).

Preliminary stability studies of liposomes loaded with propofol at 4°C and 25°C with respect to retention of the entrapped drug and changes in the size distribution were followed. Four formulations were prepared, each containing one of PC, DMPC, DPPC or DSPC. All preparations were formulated with the inclusion of 11 total molar % cholesterol, as determined during optimisation studies (Section 4.1.1).

No significant change in liposome mean volume distribution was observed between PC and its three analogous formulations for up to 28day at either 4°C or 25°C (Figures 5.4a and b, respectively). Vesicle sizes were between 5-6µm for PC and DMPC and 7-8µm for DPPC and DSPC samples throughout the course of study.

Propofol retention profiles were comparable for all four formulations at 4°C and 25°C for periods up to 28day (Figures 5.5a and b, respectively). The increase in alkyl chain length of phospholipid had no significant influence on drug retention, with all four preparations retaining majority of initially loaded propofol after completion of study. After 7day storage at 4°C and 25°C, drug retention dropped to around 80% for all samples however at time points thereafter, retention values for all four samples remained constant with no further significant losses of propofol being detected up to 28day. The 20% drop in drug retention could be attributed to unfavourable molecular arrangement of some hydrophobic propofol molecules between the hydrophilic phospholipid head-group in the bilayer. This weak molecular interaction could explain the immediate expulsion of some drug when in hydration media. Boija *et al.* (2004) state that electrostatic interactions between the drug molecules and the phospholipid head groups in the bilayer significantly affect the permeation of solutes across cell membranes.

These results suggest that alkyl chain length of phospholipid has no effect on the retention rates of the entrapped drug, which is associated with the liposomes during storage, and vesicular size, thereby suggesting all four formulations to share common stability profiles up to 28day of investigation.

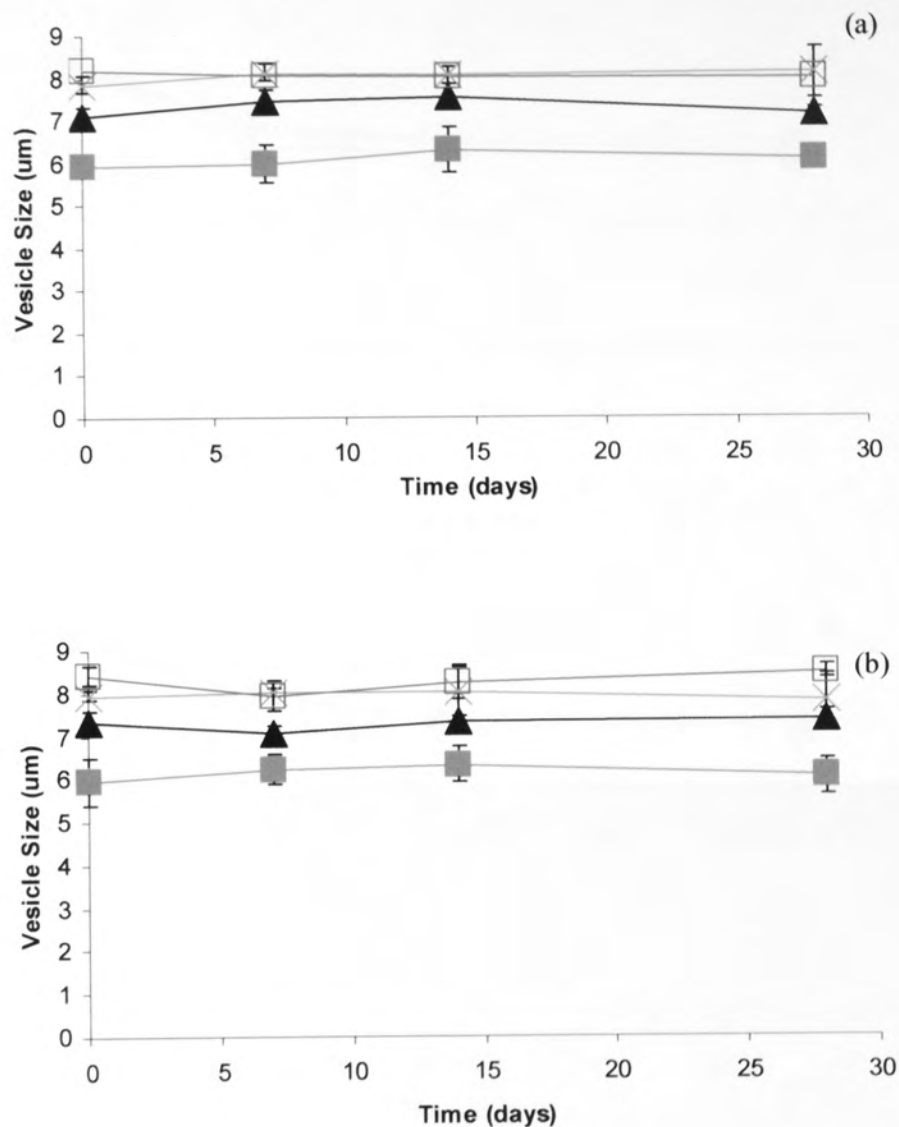


Figure 5.4 Vesicle size of PC- cholesterol (■), DMPC- cholesterol (▲), DPPC- cholesterol (⋈) and DSPC- cholesterol (□) formulations encapsulating propofol, stored at 4°C (a) and 25°C (b), for 28day. Mean volume diameter of vesicles were determined as described in Section 2.2.8. Results are expressed as the means of four experiments \pm S.D.

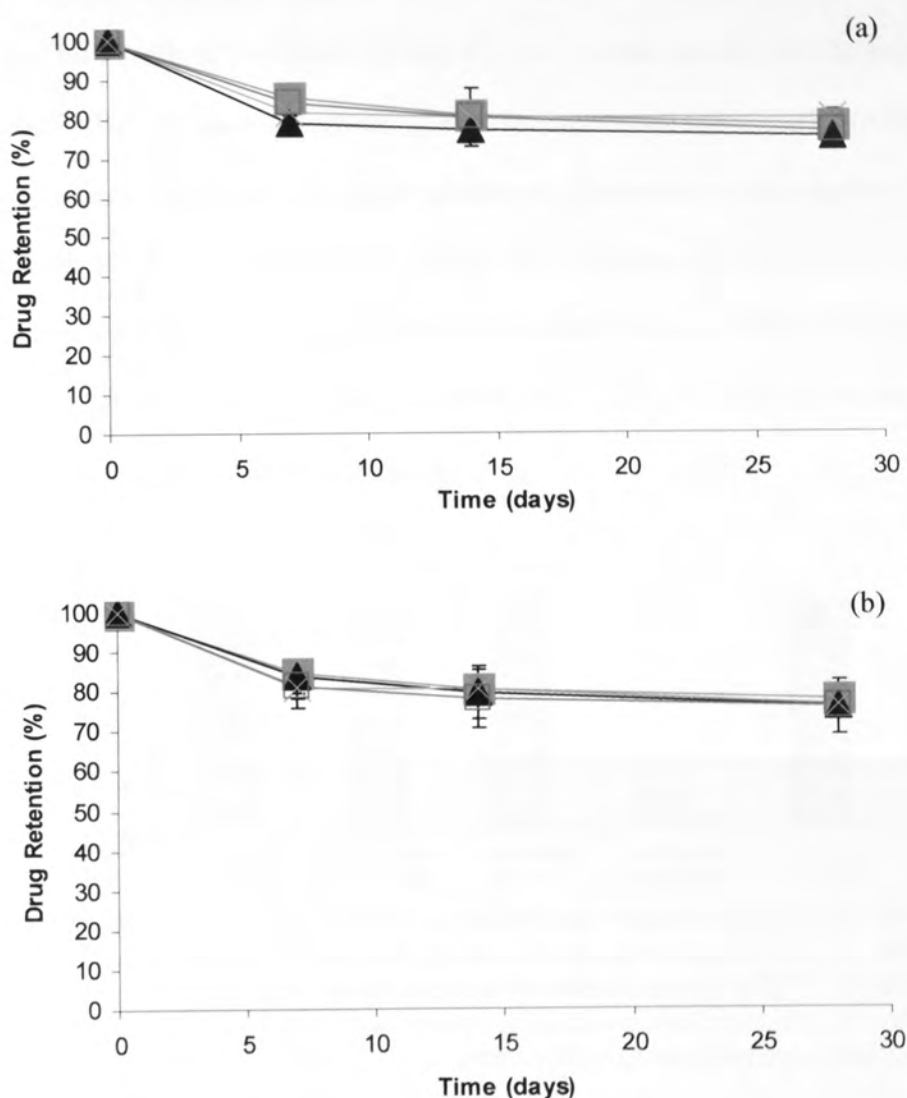


Figure 5.5 Propofol retention of PC- cholesterol (■), DMPC- cholesterol (▲), DPPC- cholesterol (x) and DSPC- cholesterol (□) formulations in aqueous buffer, pH = 7.4, stored at 4°C (a) and 25°C (b), for 28day. Propofol was encapsulated and drug retention was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27,200g for 30min. Results represent percentage retention of initially incorporated propofol. Results are expressed as the means of four experiments \pm S.D.

5.1.5 The effect of phospholipid alkyl chain length on cell toxicity

A reduction in cellular metabolic activity is a good marker of early cell damage (Chen *et al.*, 2005). The decrease of cellular metabolic activity of COS-7 cells (African green monkey kidney fibroblast cells), after exposure to liposomes, was determined *in vitro* by MTT assay at pH of 7.4 (Chen *et al.*, 2005). The influence of hydrophobic chain length on toxicity has not been sufficiently addressed to date (Lv *et al.*, 2006). Two formulations of varying length (DSPC, C₁₈ alkyl chain length; C₂₄PC, C₂₄ alkyl chain length) were studied for cell toxicity via the MTT assay.

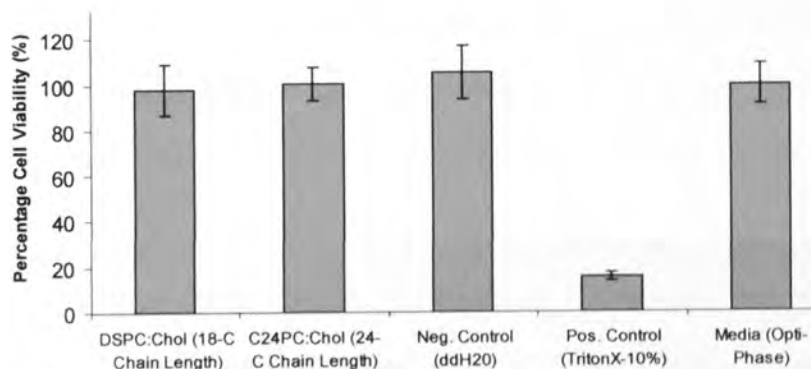


Figure 5.6 The effect of alkyl chain length of main phospholipid (DSPC or C₂₄PC) of formulation on mitochondrial dehydrogenase activity of the COS-7 cells as determined by means of the MTT assay after 4hour incubation with liposomes at 37°C. Concentration of phospholipids introduced into test system was 18μmol/mL. Results are expressed in percentage of viable cells with respect to standardised control (medium and cells) and set as mean and standard deviation, *n* = 5.

The results of the MTT assay are shown in Figure 5.6. For 4hour incubation with MLV preparations, recorded cellular activity suggest that DSPC- cholesterol and C₂₄PC- cholesterol formulations were both well tolerated by the COS-7 cells. The number of viable cells remained above 95%; therefore no statistically significant reduction in activity was observed when 18μmol/mL of either DSPC- cholesterol or C₂₄PC- cholesterol was incubated with COS-7 cells at 37°C. This suggests that the length of

phospholipid alkyl side chain up to C₂₄ has no significant bearing on cell viability. The aforementioned concentration was selected as this concentration was used to prepare formulations for encapsulation and release studies earlier (Sections 5.1.1 and 5.1.3, respectively).

5.2 The influence of lipid geometry

Many biological lipids are composed of asymmetric alkyl tail chains, where the tail moiety differs by their carbon number, presence and degree of saturation, or both (Dan, 2007; Vilcheze *et al.*, 1996). Moreover, Israelachvili *et al.* (1980) describe the packing capabilities of lipids and the eventual structures they form. They conclude that the geometry of the molecules forming these structures is critical for the formation of bilayers. Therefore it was of our interest to investigate the effect of geometry of phospholipid on hydrophobic solute encapsulation. Midazolam and propofol were selected for this investigation purely based on the fact that the former revealed one of the lowest encapsulation and the latter displaying the highest in earlier studies (Sections 4.1.1 and 5.1.1). Before this, optimisation studies were performed to assess the effect of symmetry of principle lipid component on cholesterol optimisation; only midazolam was chosen for this initial study.

5.2.1 The effect of symmetry of phospholipid on cholesterol optimisation

For comparative purposes, the symmetrical phospholipid (i.e. DMPC in Section 5.1.1) was replaced with MSPC, an asymmetrical phospholipid. MSPC has two alkyl chain moieties of differing length (C₁₄ and C₁₈) (Figure 5.7).



Figure 5.7 Structures of symmetrical DMPC (displaying its two C₁₄ alkyl chains; left) and the asymmetrical MSPC (displaying its alkyl chain moieties of differing length, C₁₄ and C₁₈; right).

Cholesterol optimisation studies showed that of those MSPC formulations tested, 11 total molar % cholesterol content and formulation without cholesterol encapsulated the highest quantity of midazolam (Figure 5.8). This is in agreement with the results seen for their symmetrical counterparts, where PC formulation containing 11 total molar % cholesterol incorporated the most amount of midazolam (Section 4.1.1). This suggests that symmetry of phospholipid has no significant bearing on the determination of optimum level of cholesterol required to achieve highest drug-loading level. The mean volume diameter and zeta potential recorded generally remained unchanged upon increase in cholesterol content (Table 5.3).

Cholesterol content (total molar %)	MSPC		PC	
	Size (μm)	ZP (mV)	Size (μm)	ZP (mV)
0.0	7.2 ± 0.4	1.2 ± 3.6	5.8 ± 0.2	-2.4 ± 5.4
11.1	7.3 ± 0.3	1.1 ± 3.2	6.2 ± 0.4	2.4 ± 5.0
20.0	7.0 ± 0.1	-2.4 ± 4.5	5.7 ± 0.3	-4.2 ± 4.8
33.3	6.4 ± 0.4	-4.3 ± 7.5	5.4 ± 0.1	-0.9 ± 6.2

Table 5.3 A comparison of the characteristics of MSPC and PC liposomes prepared using increasing amounts of cholesterol. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. Results are expressed as the means of three experiments \pm S.D. (Some data previously presented in Section 4.1, is presented here for ease of comparison)

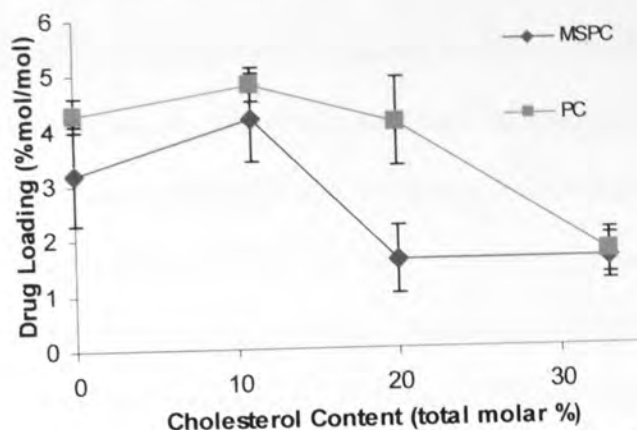


Figure 5.8 A comparison of midazolam encapsulation by MSPC and PC liposomes prepared using increasing amounts of cholesterol. The drug loading of liposomes was analysed as previously described in Section 2.2.3. Midazolam content was analysed by UV spectroscopy at 258nm wavelength. Results are expressed as the means of three experiments \pm S.D. (Some data previously presented in Section 4.1, is presented here for ease of comparison)

5.2.2 The effect of lipid geometry on liposome entrapment

The influence of lipid geometry on liposomal encapsulation of midazolam or propofol and mean volume diameter was studied. Two phospholipids, POPC and MSPC, which are structurally asymmetrical, were investigated with comparisons generated with their symmetrical counterparts, DSPC, DPPC and DMPC. Each formulation was prepared with 11 total molar % cholesterol concentration, as decided by optimisation studies (Section 5.2.1).

Liposomes encapsulating midazolam or propofol could successfully be prepared from asymmetric derivatives of PC. Studies with propofol revealed that the asymmetrical PC derivatives, MSPC and POPC, provided significantly ($P < 0.05$) lower encapsulation rates for propofol when compared to that of two of their symmetrical counterparts, DSPC and DMPC, whilst matching that of DPPC (Figure 5.9). Similarly, incorporation studies using

midazolam suggest that symmetrical PC derivatives provide more efficient encapsulation of midazolam when compared to that of their asymmetrical counterparts (Figure 5.9). The two asymmetric lipid formulations yielded the lowest encapsulation (~4 % mol/mol) therefore, suggesting regardless of nature of drug solubilised, geometry appears to play the more important role in drug bilayer loading. Statistical variance analysis showed that the midazolam-encapsulation values were significantly different for all the formulations ($P < 0.05$) except when comparing DMPC: cholesterol with MSPC: cholesterol. The generally reduced drug-loading by liposomes formulated from asymmetrical lipid maybe attributed to bilayer formation via a mismatch interaction between alkyl side-chains of asymmetric lipids, therefore reducing the overall hydrophobic area for hydrophobic drug interaction, when compared to that of symmetric lipid bilayers (Figure 5.10). The reduced drug-entrapment for MSPC vesicles could also be attributed to the single unsaturated bond it carries on the longer (C_{18}) hydrocarbon chain. Past studies (Yoshioka *et al.*, 1994) report that sorbitan monooleate (C_{9-9}) niosomes encapsulate lower amounts of solute than its alkyl analogue, C_{18} sorbitan monoester niosomes. This may be due to the fact that the presence of alkyl chain unsaturation is said to enhance chain fluidity and hence bilayer permeability (Uchegbu and Florence, 1995; Devaraj *et al.*, 2002). Investigations using a Langmuir trough by Hac-Wydro and Wydro (2007) showed that the inclusion of a saturated fatty acid to a phospholipid/cholesterol model membrane makes the model system more rigid, whereas the presence of an unsaturated fatty acid enhances its fluidity. Additionally, it is recognised that cholesterol has lower solubility in membranes with polyunsaturated acyl chain moieties (Benatti *et al.*, 2007; Brzustowicz *et al.*, 2002). This can be rationalised by the fact that polyunsaturated chains display a jagged shape that

does not pack adequately with the smooth surface of cholesterol (Benatti *et al.*, 2007), thereby not rigidifying a bilayer as well as sufficiently-packed cholesterol in a saturated bilayer would.

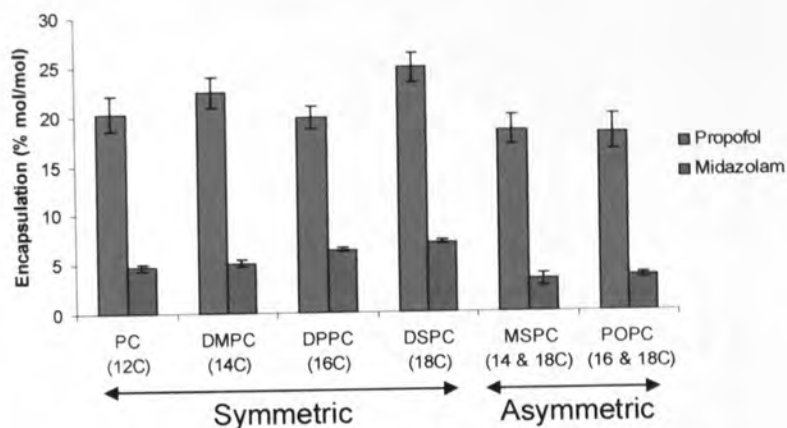


Figure 5.9 The influence of long-chain symmetric and asymmetric lipids on midazolam or propofol encapsulation. Liposomes were prepared from a combination of PC, DMPC, DPPC, DSPC, POPC or MSPC with 11 total molar % cholesterol and drug loading in a total lipid concentration of 18 μ mol. The drug content was analysed by UV spectroscopy at 258nm (midazolam) or 268nm (propofol) wavelengths. Results are expressed as the means of at least four independent experiments \pm S.D.

The sizes obtained for liposomes prepared from asymmetrical lipids encapsulating midazolam or propofol were significantly lower than those obtained for DSPC vesicles. For example, size measurements of 7.23 ± 0.16 and 7.27 ± 0.29 obtained for midazolam-loaded POPC and MSPC formulations respectively (Table 5.4) were significantly smaller than that for DSPC preparation (8.43 ± 0.19 ; Section 5.1.1). This is interesting since both asymmetric lipids possess a C₁₈ alkyl side-chain that is akin to the two attached to the phosphate head-group of DSPC. This could also support the theory of a reduction in the overall bilayer hydrophobic region for the lipophilic midazolam to interact with thus yielding decreasing loading rates (Figure 5.9). Zeta potential did not show any relation with geometry (Table 5.4).

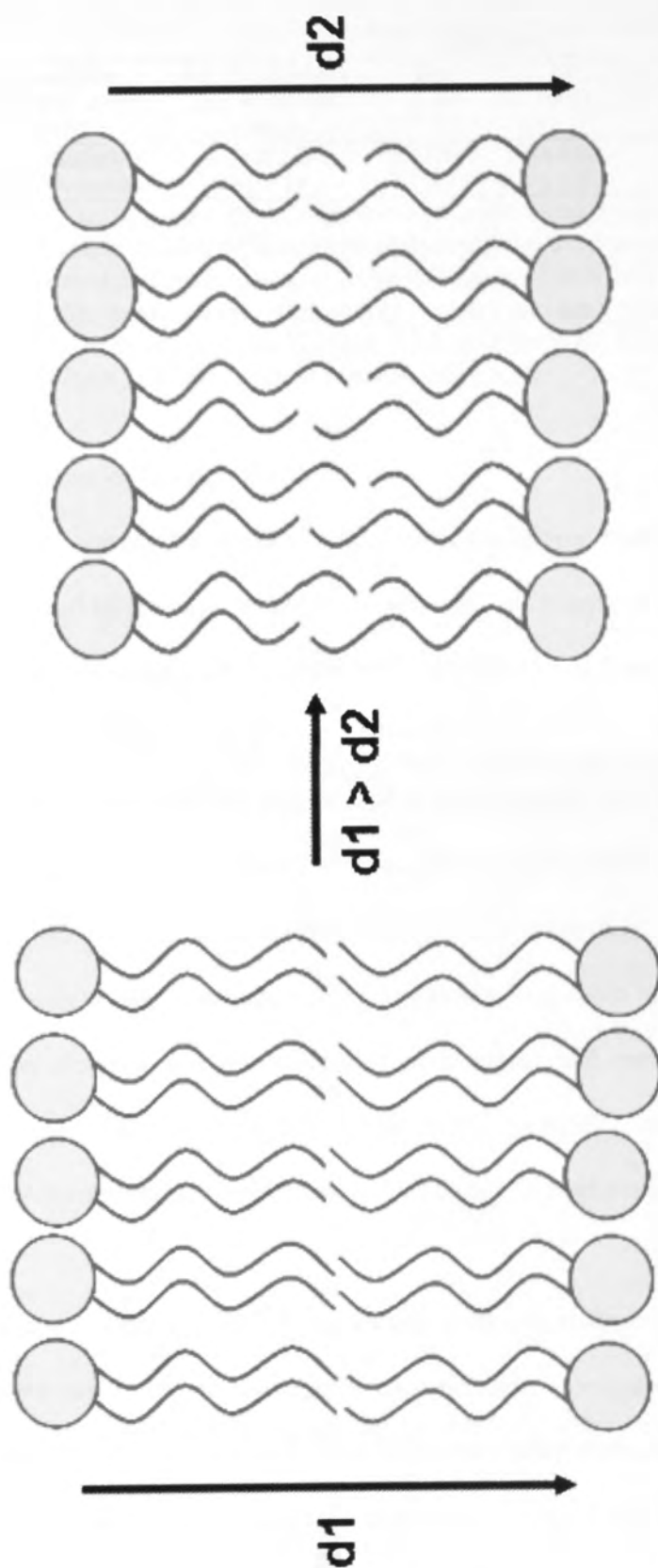


Figure 5.10 Diagram proposing the possible loss of hydrophobic region in an asymmetric lipid bilayer due to a 'mismatch' interaction between phospholipids possessing two hydrocarbon side-chains of differing length resulting in diameter reduction (right) when compared to a bilayer containing a symmetrical lipid (left). (d = diameter)

Formulation (0.89: 0.11 molar ratio)	Lipid tail length (no. of carbon atoms)	Midazolam		Propofol	
		Size (μm)	ZP (mV)	Size (μm)	ZP (mV)
POPC: cholesterol	16 & 18	7.2 ± 0.2	6.4 ± 6.1	6.4 ± 0.4	-1.2 ± 3.1
MSPC: cholesterol	14 & 18	7.3 ± 0.3	1.1 ± 3.2	8.2 ± 0.4	1.9 ± 2.4

Table 5.4 *The influence of asymmetric lipids on mean volume diameter and vesicle charge. Characterisation of liposomes prepared from a combination of POPC or MSPC with cholesterol and midazolam or propofol encapsulation in a total lipid concentration of 18 μmol . The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. Results are expressed as the means of at least four independent experiments \pm S.D.*

5.2.3 Combination Studies

Past studies suggest that physical properties of the bilayer, such as fluidity and thickness, can be altered by creating 'voids' in the phospholipid bilayer. Kragh-Hansen *et al.* (1998) discovered that when C_{12}NO (a non-ionic surfactant) was formulated with phospholipid, voids ('free volume') were created within the bilayer due to hydrophobic mismatch via the interaction between the shorter C_{12}NO hydrocarbon chain and longer phospholipid alkyl chain. It was therefore interesting to study the influence a potential mismatch at the bilayer level would have on the encapsulation of a hydrophobic model drug candidate as well as vesicle size. The influence of combining long-chain symmetric and asymmetric lipids on incorporation of midazolam or propofol and mean volume diameter was investigated. DSPC was combined with POPC or MSPC, and DPPC with POPC or MSPC. All formulations were prepared containing 11 total molar % cholesterol.

Combining a symmetric lipid with its asymmetric counterpart yielded interesting results. A notable improvement in propofol encapsulation (compared to their respective uncombined lipids) was observed when liposomes were formulated from a mixture of a symmetric lipid and an asymmetric derivative (Figure 5.11). However, the effect was

reversed when propofol was substituted for an alternative drug. Combining long-chain symmetric and asymmetric lipids on midazolam encapsulation yielded less efficient formulations displaying a significant reduction in midazolam encapsulation, when compared to entrapment shown by un-combined lipid formulations (Figure 5.11). This suggests that possible 'voids' created in the bilayer (as aforementioned) by a mismatch arrangement of phospholipids can enhance solubilisation of certain hydrophobic drugs while reducing the effect of others.

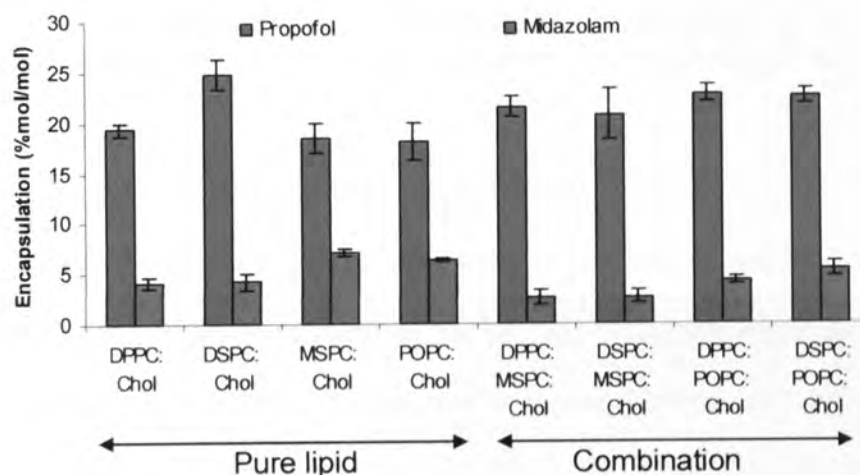


Figure 5.11 The influence of combining long-chain symmetric and asymmetric lipids on drug encapsulation. Liposomes were prepared from a combination of DSPC or DPPC with POPC or MSPC and cholesterol with drug encapsulation in a total lipid concentration of $18\mu\text{mol}$. Drug loading of vesicles was determined as stated in Section 2.2.3. The drug content was analysed by UV spectroscopy at 258nm (midazolam) or 268nm (propofol) wavelengths. Results are expressed as the means of at least four independent experiments \pm S.D. (Results for the single-lipid based formulations were presented earlier in Section 5.2.2 and is used here for ease of comparison)

Results observed for midazolam- or propofol- loaded formulations revealed no significant difference between the mono-lipid based preparations and the combined versions with respect to vesicle size and overall surface charge (Table 5.5).

Formulation	Composition (molar ratio)	Midazolam		Propofol	
		Size (μm)	ZP (mV)	Size (μm)	ZP (mV)
DPPC: cholesterol	0.89: 0.11	7.5 ± 0.1	-4.1 ± 3.2	8.7 ± 1.0	-3.6 ± 1.5
DSPC: cholesterol	0.89: 0.11	8.4 ± 0.2	-1.9 ± 3.6	8.5 ± 1.1	-4.4 ± 3.7
MSPC: cholesterol	0.89: 0.11	7.3 ± 0.3	1.1 ± 3.2	8.2 ± 0.4	1.9 ± 2.4
POPC: cholesterol	0.89: 0.11	7.2 ± 0.2	6.4 ± 6.1	6.4 ± 0.4	-1.2 ± 3.1
DPPC: MSPC: cholesterol	0.44: 0.44: 0.11	7.7 ± 0.3	2.8 ± 4.3	7.7 ± 0.4	-6.8 ± 1.4
DSPC: MSPC: cholesterol	0.44: 0.44: 0.11	7.1 ± 0.8	6.1 ± 2.4	6.3 ± 0.6	3.6 ± 1.9
DPPC: POPC: cholesterol	0.44: 0.44: 0.11	6.1 ± 0.6	2.9 ± 4.5	7.7 ± 0.5	-2.4 ± 4.6
DSPC: POPC: cholesterol	0.44: 0.44: 0.11	10.3 ± 0.5	-5.3 ± 3.3	7.1 ± 0.3	3.3 ± 1.4

Table 5.5 The influence of combining long-chain symmetric and asymmetric lipids on mean volume diameter and vesicle surface charge. Characterisation of liposomes prepared from a combination of DSPC or DPPC with POPC or MSPC and cholesterol with propofol encapsulation in a total lipid concentration of $18\mu\text{mol}$. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. Results are expressed as the means of at least four independent experiments \pm S.D. (Results for the single-lipid based formulations were presented earlier in Sections 5.11 and 5.22)

5.2.4 The effect of phospholipid symmetry on cell toxicity

Earlier, variation in alkyl chain length was found not to induce or affect cellular toxicity (Section 5.15). For comparative purposes, the decrease of cellular metabolic activity of COS-7 cells, following exposure to liposomes formulated from phospholipids of varying geometrical orientation, was determined *in vitro* by MTT assay at pH of 7.4. Two formulations of differing symmetry (DSPC, symmetric; MSPC, asymmetric) were studied relative to standards for cell toxicity via detection of the quantity of formazan produced.

The results of the MTT assay are shown in Figure 5.12. For 4hour incubation with MLV preparations, recorded cellular activity suggest that DSPC-cholesterol and MSPC-cholesterol formulations were both well tolerated by the COS-7 cells. The number of viable cells remained above 95%; therefore no statistically significant reduction in activity was observed when $18\mu\text{mol/mL}$ of either DSPC-cholesterol or MSPC-cholesterol

was incubated with COS-7 cells at 37°C. This suggests that the geometric status of phospholipid chain has no significant bearing on cell viability.

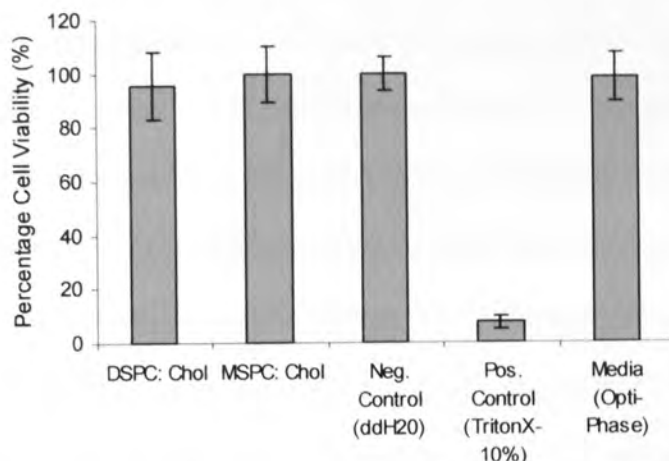


Figure 5.12 A comparative study of mitochondrial dehydrogenase activity of the COS-7 cells by DSPC and MSPC as determined by means of the MTT assay after 4 hour incubation with liposomes at 37°C. Concentration of phospholipids introduced into test system was 18 $\mu\text{mol/mL}$. Results are expressed in percentage of viable cells with respect to standardised control (medium and cells) and set as mean and standard deviation, $n = 5$.

5.3 The influence of alkyl chain length and symmetry in monolayers at the air/water interface

Initially, the effect of alkyl chain length was examined with various phospholipids having identical polar head-group, however differing in their hydrophobic alkyl chain length; namely DSPC, DPPC and DMPC were selected (Dynarowicz-Latka and Hac-Wydro, 2004). The compression surface pressure-area per molecule isotherms of pure compounds are plotted in Figure 5.13. Generally, the surface pressure for the transition from liquid-expanded to liquid-condensed phase increased with decreasing chain length (Barnes and Gentle, 2005). For DSPC, the transition from gaseous to liquid expanded phase occurred at an approximate molecular area of $58 \text{ \AA}^2/\text{molecule}$ while the liquid-expanded to liquid-

condensed shift transpired at surface pressure of around 5mN/m. The film eventually collapsed at ~60mN/m. The DPPC isotherm revealed a more expanded shape with gaseous to liquid-expanded transition taking place at ~ 95Å²/molecule. The switch from liquid-expanded to liquid-condensed occurred at surface pressure of approximately 10mN/m with monolayer finally collapsing at about 65mN/m. The DMPC isotherm was the least condensed and reveals that this lipid is in a liquid-expanded phase at the start of the compression process (~125Å²/molecule) and thereon converts to the liquid-condensed phase at around a surface pressure of 18-20mN/m. The collapse pressure for DMPC is the lowest (around 55mN/m) compared to DSPC and DMPC suggesting the lipid to be least stable as stability linearly correlates with the pressure at point of collapse (Dynarowicz-Latka and Hac-Wydro, 2004). The limiting mean molecular area for DSPC, DPPC and DMPC were 51.0, 55.8 and 65.5Å²/molecule, respectively (Table 5.6). The trend could be attributed to degree of cohesion between the chains. In this homologous series of long-chain compounds, the attraction between the chains will dictate how closely packed they arrange themselves. DSPC being the longest will translate in the existence of the strongest chain-chain interactions resulting in closely packed chains (Gaines, 1966); this being reflected in its isotherm being nearly straight and steep. The longer the chain the greater is the Van der Waal's attraction allowing the lipid molecules to cohere strongly, thereby condensing the isotherm (Hac-Wydro *et al.*, 2007). The molecules of DSPC are packed with more compactness than those of DPPC (Hac-Wydro *et al.*, 2007). With decreasing chain length (i.e. DMPC < DPPC < DSPC), this cohesion depreciates yielding films that become more 'gaseous' (Davies and Rideal, 1963). This work supports the rational of longer chain alkyl lipids producing more stable liposomes, since the longer

alkyl chains, with stronger cohesion, resulting in less leakage from the aqueous core of liposomes has been previously reported (e.g. Mohammed *et al.*, 2004 and Taylor *et al.*, 1990). With reference to drugs incorporated within the liposomal membranes, this could support longer retention within the bilayers (as shown in Section 5.1.3) if good cohesion between the lipids is supported.

Symmetry of phospholipids was also examined using Langmuir monolayer studies. The isotherm for MSPC which has an average alkyl chain carbon number of 16 (akin to that of DPPC) bears a strong resemblance to that of DPPC (Figure 5.13); gaseous to liquid-expanded transition occurred at mean molecular area $\sim 95 \text{ \AA}^2/\text{molecule}$ with monolayer collapsing at surface pressure of about 65 mN/m (Table 5.6). However the changeover from liquid-expanded to liquid-condensed withstood greater surface pressures compared to DPPC and arose at approximately 18 mN/m. The similarities in these features further suggest that Van der Waal's interaction is a key parameter in deducing monolayer effects at the air/water interface.

POPC is another asymmetrical phospholipid that was investigated. The lipid contains a 16 and 18-carbon alkyl chain, with a sole unsaturated bond (in *cis*-configuration). Since the average carbon number for each alkyl chain is 17 for POPC, one would expect that the lipid should behave like something lying in between the air/water behaviour of DSPC and DPPC. Analysing the isotherm (Figure 5.13) for POPC, it is evident that the presence of unsaturation leads to the formation of a less condensed monolayer when weighed against saturated phospholipids of similar alkyl chain length. In the case of condensed

film (such as those reported for the homologous series above) the chain-chain interactions are of immense significance. Only molecules which have the capability of exceedingly intimate contact can display such an interaction (Dynarowicz-Latka and Hac-Wydro, 2004). The introduction of a double bond could alter this packing possibility (Gaines, 1966) as it may have in the case of POPC. The unsaturated bond in the 18-carbon alkyl chain bends the mono-unsaturated alkyl group away from the vertical 16-carbon chain. During compression, the protruding alkyl chain induces steric hindrance thus increasing the distance between the molecules making close matching much more difficult. It is established that the presence of *cis* double bonds in the hydrophobic alkyl chain influences its geometrical structure, with the actual number of these bonds present being proportional to how bent the chain will exist (Hac-Wydro and Wydro, 2007). In the situation of POPC, the increase in intermolecular distance results in the reduction of Van der Waal's attractions between their hydrophobic parts, therefore influencing the condensation of monolayer, lowering the collapse pressure rendering the membrane less stable (Hac-Wydro and Wydro, 2007).

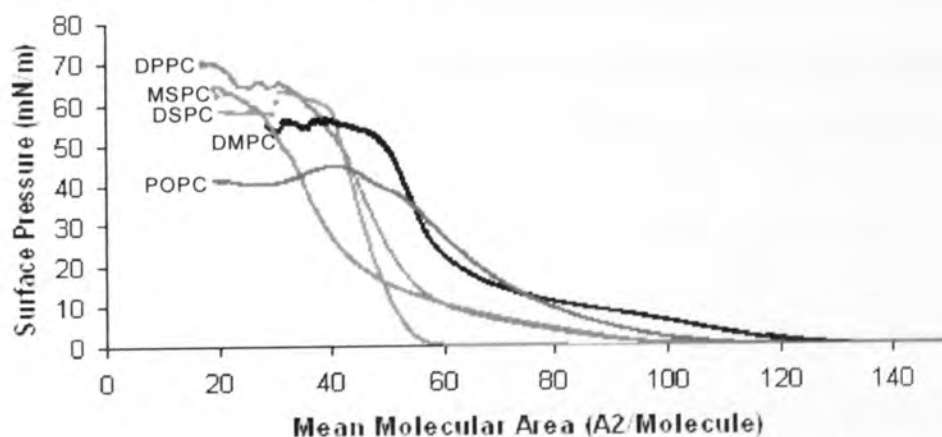


Figure 5.13 The surface pressure-area isotherms of pure monolayers of DSPC, DPPC, DMPC, MSPC and POPC at the air/water interface (at 20°C). Results are expressed as the means of three experiments \pm S.D.

In conclusion, the presence of double bond dictates Van der Waal's whilst the absence of unsaturation means that the carbon number predominates. This suggests that alkyl chain length alone does not dictate stability of liposomal formulations; the geometric features of the chain moieties also play an important role in the eventual vesicle stability profile.

Lipid category	Lipid	Extrapolated area at zero pressure (Å ² /molecule)	Collapse pressure (mN/m)
Symmetric	DSPC	51.0	63.2
	DPPC	55.8	64.3
	DMPC	65.5	56.2
Asymmetric	MSPC	48.5	63.5
	POPC	80.8	44.9

Table 5.6 Extrapolated area and collapse pressure of pure DSPC, DPPC, DMPC, MSPC and POPC monolayers at the air-water interface at temperature of 20°C. Results are expressed as the means of three experiments.

5.4 Effect of drug properties on vesicle characteristics

It is well established that the physiochemical characteristics of liposomes (e.g. bilayer composition, size, and surface charge) affect drug loading and release (e.g. Mohammed *et al.*, 2004). Thereby, if carrier systems are being prepared to solubilise hydrophobic drugs, it is imperative to understand how the properties of drugs (e.g. Log P and MW) will alter vesicle drug loading. For example, drug molecules can significantly alter fundamental features of vesicles such as size, surface charge and bilayer integrity (Fatouros and Antimisariis, 2002).

To investigate the influence of drug features on drug incorporation in liposomes composed of PC and cholesterol (0.89: 0.11 molar ratio), a range of drugs (diazepam, ibuprofen, midazolam, phenytoin, propofol and rifampicin) were investigated. The characteristics of these drugs are outlined in Section 3.0.

Drug lipophilicity is expressed by Log P, the logarithm of the octanol-water partition coefficient and it is accepted to correspond to the general affinity of a chemical to partition between an organic and an aqueous phase (Momo *et al.*, 2005). When encapsulation values were plotted against the drug lipophilicity, no apparent trend was observable ($R^2 = 0.081$; Figure 5.14a), at least for the range of drugs considered here. Conversely, plotting MW against encapsulation showed a degree of linear correlation when rifampicin was excluded from the picture ($R^2 = 0.86$) (Figure 5.14b).

Propofol (MW of 178Da) displayed the highest bilayer incorporation of 20.3 % mol/mol whilst rifampicin (MW of 823Da) revealed the least. This could be attributed to decreasing effectiveness in bilayer packing with increasing MW. Molecular size (which generally correlates with MW) is stated to be an important factor with respect to how drugs arrange themselves within the bilayer (Fatouros and Antimisariis, 2002). For example, Balasubramanian and Straubinger (1994) reported unfavourable bilayer packing by paclitaxel, which was cited to be due to the bulky structure of the drug.

For similar lipophilicity values, i.e. 3.48, 3.87 and 3.84 (ibuprofen, midazolam and, propofol, respectively; Wishart, 2006), encapsulation significantly increased in the order of propofol > ibuprofen > midazolam reflecting their decreasing MW. Therefore it is very possible that MW plays the more influential role in dictating levels of drug encapsulation as opposed to drug lipophilicity, which yielded no clear correlation with respect to bilayer drug entrapment.

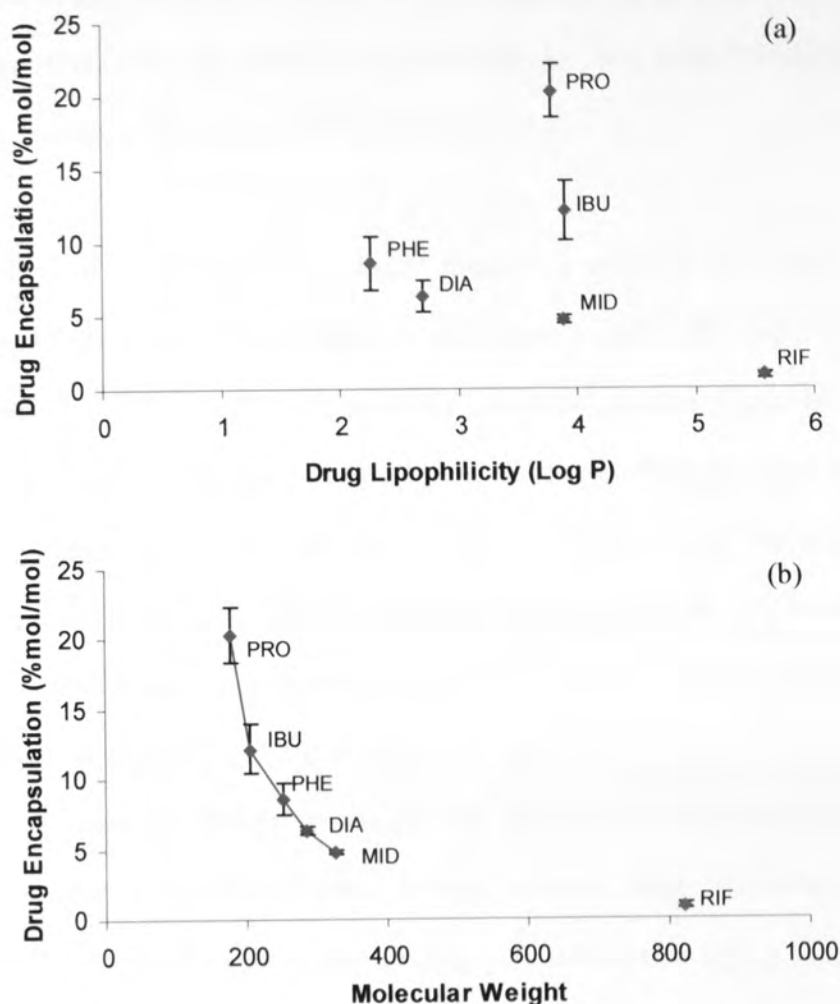


Figure 5.14 The relationship between liposomal (PC: cholesterol, 0.89: 0.11 molar ratio) bilayer encapsulation of drug and a) lipophilicity or b) molecular weight of drug. Results are expressed as the means of at least four experiments \pm S.D.

5.5 Charged lipid studies

5.5.1 The influence of charged lipids on liposomal-incorporation of propofol

Liposomes were prepared from cationic lipids (DSTAP, C_{18} alkyl chain length; DPTAP, C_{16} alkyl chain length; DMTAP, C_{14} alkyl chain length) and their physico-chemical properties were compared against their neutral phospholipid analogues, with respect to

alkyl chain length (DSPC, DPPC, and DMPC, respectively), to potentially yield near-fully charged vesicles. All formulations contained 11 total molar % cholesterol and propofol (the highest encapsulated drug in Section 5.1.1).

Investigations of positively-charged lipids revealed a trend of increasing liposome-entrapment of propofol with an increase in alkyl chain length in the order of DSTAP > DPTAP > DMTAP ($P < 0.05$) (Figure 5.15a). This trend appears to correlate with data obtained for their corresponding neutral lipid derivatives where the trend of $C_{24}PC > DSPC > DMPC \geq DPPC = PC$ was seen ($P < 0.05$) (Section 5.1.1). The results yielded suggest that, irrespective of the surfactant type, the solubility of a hydrophobic drug increases with increasing surfactant chain length. A similar conclusion was published by Rangel-Yagui *et al.* (2005) who investigated the solubilisation of ibuprofen in micellar solutions of three surfactants possessing the same alkyl tail length but variable hydrophilic head groups (i.e. sodium dodecyl sulphate, dodecyltrimethylammonium bromide and *n*-dodecyl octa(ethylene oxide)) with results showing an increase in the solubility of the poorly water-soluble drug in linear fashion with increasing surfactant content for all surfactants.

Interestingly, results revealed a significant ($P < 0.05$) general improvement in propofol-encapsulation by near fully positively-charged liposomes when weighed against their neutral counterparts. DSTAP and DPTAP encapsulated a significantly greater quantity of drug than their neutral analogues, DSPC and DPPC, respectively, with DMTAP revealing no significant difference in propofol-loading when weighed against DMPC. The increase

in drug-incorporation by the fully charged liposomes could be due to intermolecular π -cation interaction between the aromatic ring of propofol and the positively charged nitrogen on the head-group of the TAP structures; a non-bonded interaction that has attracted considerable attention (Garau *et al.*, 2004). Benzene is non-polar since no single carbon atom is more negatively charged than the other carbon atoms; there is no net dipole moment. However, the distribution of electrons is uneven throughout the molecule resulting in a state termed 'quadrupole' where there exists more negative charge both above and below the hexagonal plane of carbons (Ma and Dougherty, 1997). Empirical evidence suggests that this kind of interaction exist and is relatively strong (Garou *et al.*, 2004). X-ray crystal analysis by Hamada *et al.* (2006) reveal the molecular assembly of thiacalix[6]arene via π -cation interaction between the aromatic ring of thiacalix[6]arene and the positively charged group such as nitrogen of dimethylformamide. Furthermore, Dougherty *et al.* (1990) proposed that the π -cation interaction is responsible for the binding of acetylcholine to its deactivating enzyme, acetylcholinesterase. Puglisi *et al.* (1995) associate the binding of drugs to liposomes to ionic bonding between negatively charged phosphate moiety of the phospholipid and the positively charged piperazine ring pertaining to fluoroquinolone.

Near-fully charged anionic MLV were prepared from the negatively charged 1,2-distearoyl-3-phosphatidylglycerol (DSPG, 18-carbon alkyl chain length; Figure 5.16) for comparative effect. Propofol-loading by DSPG liposomes was significantly lower than for their 18-carbon neutral DSPC and cationic DSTAP analogous formulations (Figure 5.15a). This could be due to repulsive interaction between the anionic head-group of

DSPG and the electron-strong aromatic moiety of propofol. Overall positively charged liposomes exhibited the highest encapsulation efficiency, followed by neutral ones, and then by negatively charged liposomes, using the same lipid: cholesterol molar ratio. A similar trend has been cited by Hathout *et al.* (2007) with the group attributing this behaviour to acetazolamide being a weak acid, creating an electrostatic attraction between drug anion and the positively charged stearylamine thus accounting for the higher encapsulation efficiency when compared with the negatively charged vesicles, where such interaction would not be possible; whilst in contrast, in case of negatively charged liposomes, it is probable that charge repulsion may transpire between the drug molecules and the negatively charged dicetyl phosphate, consequently suppressing the loading percentage.

As expected, the cationic liposomes exhibited zeta potential values of around 40mV and the anionic formulation recorded a figure of around -80mV (Figure 5.15b), therefore confirming the charged nature of the respective liposomal surfaces.

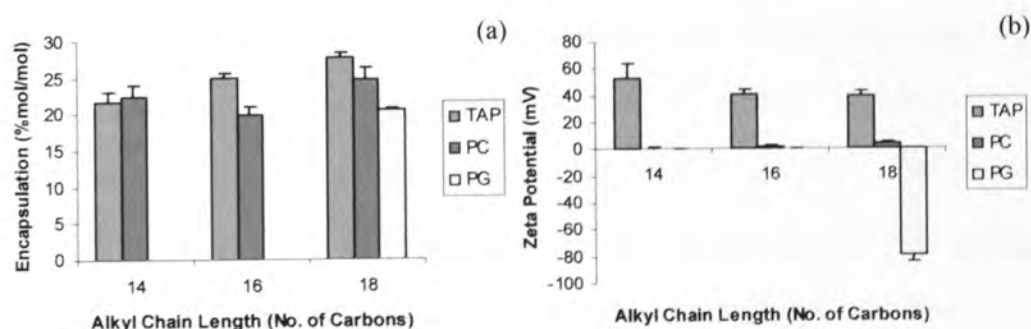


Figure 5.15 Encapsulation of propofol by charged liposomes weighed against their neutral phospholipid analogue (a). The vesicle surface charge of charged vesicles compared against the zeta potential of their neutral counterpart (b). Results are expressed as the means of at least four independent experiments \pm S.D.

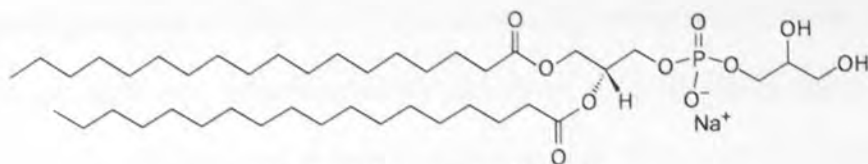


Figure 5.16 Structure of DSPG.

TEM was utilised to study the morphological characteristics of liposomes suspended in double-distilled water. TEM images (example in Figure 5.17a) revealed that the cationic liposomes were discrete, spherical vesicles. ESEM Images (images shown in Section 7.4) collected from analysis of liposomes made from the PC analogues DSPC, DPPC and DMPC and TEM micrographs (example for DSPC formulation shown in Figure 5.17b) revealed notable signs of partial vesicular aggregation, an outcome previously reported. Drummond *et al.* (1999) state that although liposomes composed of DSPC-cholesterol release drug very slowly, they are difficult to work with owing to enhanced aggregation and flocculation over time.

The coalescing effect reduced upon the introduction of cationic charge in the liposomal bilayer as would be expected; liposomes prepared from charged lipids (i.e. DMTAP, DPTAP and DSTAP) generally appeared to show less vesicular aggregation as when compared to their neutral counterparts (i.e. DMPC, DPPC and DSPC) probably due to repulsion between positively charged vesicles (an example of such a comparison in Figure 5.17). The influence of charge on vesicle dispersion is well established. A report by du Plessis *et al.* (1996) stated that the presence of charged lipids decreases the likelihood of aggregation following synthesis. In another study, Zhang *et al.* (2005) concluded that the addition of a negatively charged lipid (cardiolipin) significantly

prevented aggregation and fusion of liposomes due to electrostatic repulsion. In lyophilic colloids and emulsions, repulsive electrostatic forces are a stabilising factor (Alonso *et al.*, 1995). The stability in a colloidal system such as liposomes is described by the DLVO theory. The theory, based on attractive Van der Waal's and repulsive electrostatic interactions, describes the force between charged surfaces interacting via an aqueous medium (Malmsten *et al.*, 1996). Therefore, at a certain concentration of electrolyte, it is possible to reach a maximum in the zeta potential and the lowest aggregation of colloidal particles thereby increasing colloidal stability (Alonso *et al.*, 1995; Malmsten *et al.*, 1996). TEM studies (Figure 5.17) revealed no significant difference in vesicle sizes between neutral and charged formulations.

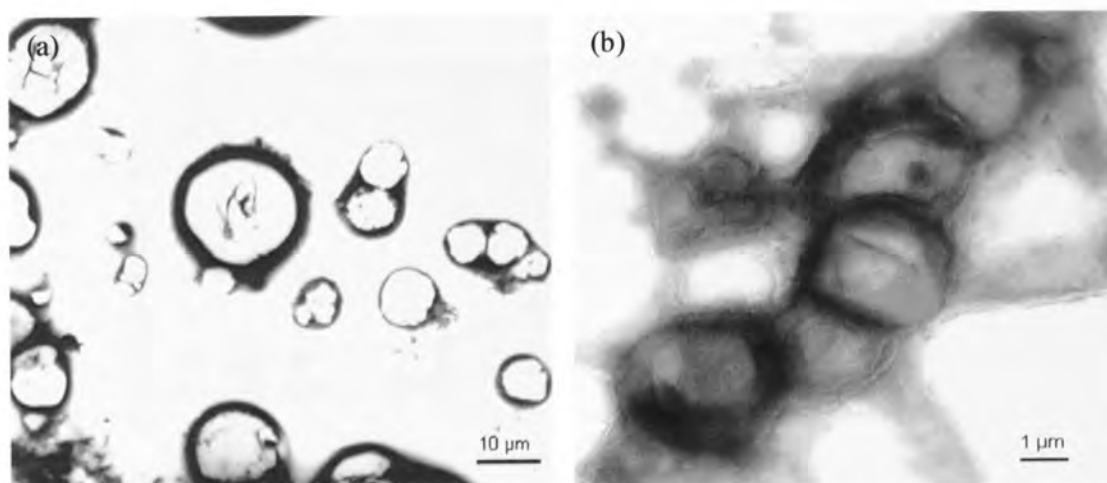


Figure 5.17 TEM micrographs of liposomes encapsulating propofol (in double-distilled water): DSTAP (0.89: 0.11 molar ratio) vesicles (a) showed a significant reduction in aggregation compared to corresponding neutral DSPC- cholesterol (0.89: 0.11 molar ratio) preparation (b).

5.5.2 The release of propofol from charged liposomes

Figure 5.18 demonstrates that the cumulative drug amount released at 72hour varied between the DSPC, DSTAP and DSPG preparations. Of the three, drug release from

DSPC preparation cumulatively released the least with 50% of propofol released after 72hour. On completion of the study (72hour), drug loss from DSTAP liposomes was the highest (~90%) whilst DSPG vesicles released just over 80% of drug, rendering the former a significantly faster ($P < 0.05$) drug-releasing vehicle.

The effect of charge on drug permeability has been previously documented; results collated by Nounou *et al.* (2006) revealed that positively charged liposomes exhibited the fastest release compared with neutral and negatively charged preparations. Furthermore, Mohammed *et al.* (2004) demonstrated that the inclusion of 9 total molar % stearylamine (positively charged) or dicetylphosphate (negatively charged) induced significantly more release of ibuprofen compared to neutral liposomes.

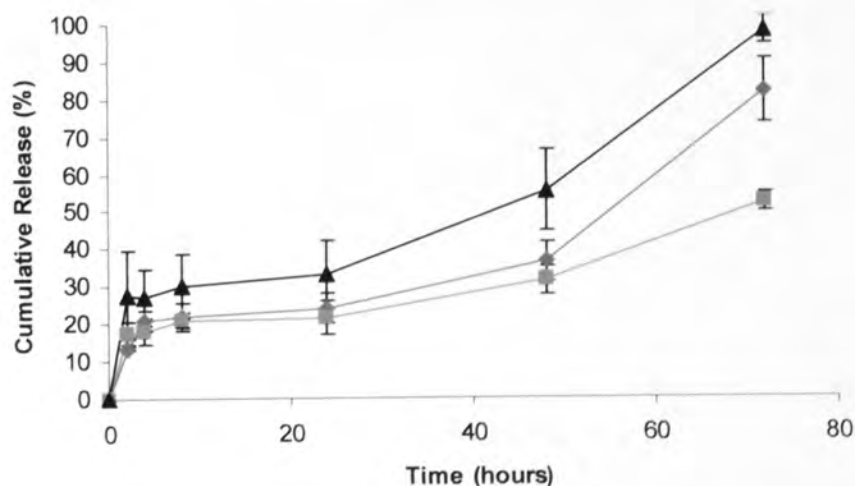


Figure 5.18 Effect of vesicle surface charge on rate of propofol release. Propofol was encapsulated and drug release from DSPC (■), DSTAP (▲) and DSPG (◆) was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27,200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments \pm S.D. (Data for DSPC formulation was previously presented in Section 5.1.3, is presented here for ease of comparison)

Further to the investigations in Section 5.1.3, alkyl chain length of TAP series was also probed. Three formulations consisting of DMTAP, DPTAP or DSTAP and including 11 total molar % cholesterol were incubated in PBS at 37°C for up to 72hour and their release of retained propofol examined. Results recorded (Figure 5.19) suggest that alkyl chain length of TAP does not appear to influence the cumulative release of propofol. This is in contrast to the outcome observed for neutral lipid formulations in Section 5.1.3. This suggests that in case of a fully charged bilayer, as yielded by the lipids stated here, overall bilayer charge appears to dictate permeability as opposed to lipid transition temperature and thus alkyl chain length (as appears to be the situation for neutral formulations; Section 5.1.3).

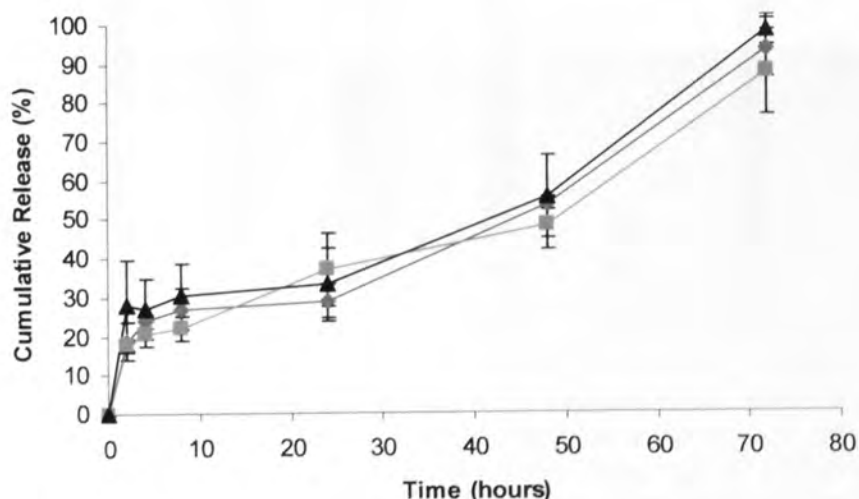


Figure 5.19 Effect of alkyl chain length of positively charged TAP series on bilayer release of propofol. Propofol was encapsulated and drug release from DMTAP (◆), DPTAP (■) and DSTAP (▲) was measured on the basis of untrapped drug recovered in the suspension following centrifugation at 27,200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments \pm S.D. (Data for DSTAP formulation was previously presented in Figure 5.18, is presented here for ease of comparison)

5.5.3 The effect of charge on cell toxicity

A decrease in cellular metabolic activity is an established indicator of early cell damage (Chen *et al.*, 2005). The effect of phospholipid head-group charge on the metabolic activity of COS-7 cells was examined *in vitro* via the MTT assay at physiological pH. Evidence collected in the past (Lappalainen *et al.*, 1994; Lincopan *et al.*, 2006; Vangala *et al.*, 2006) report cationic lipids to be toxic. The cationic lipid, DSTAP was compared with a neutral (DSPC- cholesterol) and a negative (DSPG- cholesterol) counterpart. Each phospholipid harbours two alkyl chain groups that are 18-carbon in length and all preparations were produced in 0.89: 0.11, lipid: cholesterol molar ratio.

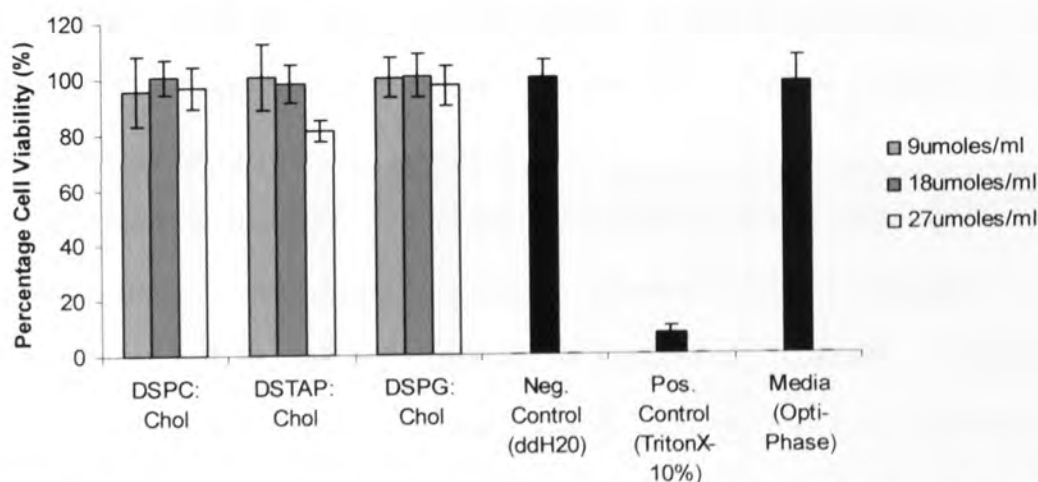


Figure 5.20 Effect of bilayer charge and lipid concentration on the percentage cell viability of COS-7 cells. Mitochondrial dehydrogenase activity of the COS-7 cells determined by means of the MTT assay after 4hour incubation with liposomes at 37°C. Concentration of phospholipids introduced into test system was 9-27 μmol/mL. Results are expressed in percentage of viable cells with respect to standardised control (medium and cells) and set as mean and standard deviation, $n = 5$.

Results obtained revealed no marked reduction of cellular activity compared to controls regardless of lipid head-group charge (Figure 5.20), therefore implying the stated lipids resulted in no measurable toxicity at the above-mentioned concentrations. To further

examine this, concentration of all three lipids exposed to the COS-7 cells were increased in order to obtain a threshold level for significantly reduced cellular activity. Increased concentrations up to 27 $\mu\text{mol/mL}$ of DSPC and DSPG were tolerated by the cells with no apparent decrease in metabolic activity when compared to standard (Figure 5.20). In the case of DSTAP, a 20% reduction in activity was seen at lipid concentration of 27 $\mu\text{mol/mL}$, with maximum tolerated concentration appearing to be at 18 $\mu\text{mol/mL}$.

The results obtained above using the MTT assay highlighted the utilisation of the well-established (Borenfreund *et al.*, 1988; Kapitza *et al.*, 2007) MTT assay to assess toxicity of test substances, in this case, liposomes. This method was compared to a second *in vitro* method, the Red Blood Cell (RBC) assay. This haemolysis method examines the membrane-disruptive action of liposomes via the photometric detection of oxyhaemoglobin following RBC lysis (Chen *et al.*, 2005; ECVAM, 1992). RBC are recognised to be an excellent source for examining influence on biological membranes: the cells are homogenous and their simplicity in handling for standard testing and quantification allows them to be used routinely. The RBC assay is rapid, inexpensive and yields reliable results with good reproducibility, diminishes and even circumvents the use of experimental animals for tests of this nature (Lagarto *et al.*, 2006). Samples (DSPC: cholesterol, DSTAP: cholesterol and DSPG: cholesterol) prepared in the isotonic buffer, PBS, were incubated with an appropriate quantity of RBC suspension, centrifuged and absorbance of supernatant analysed. Results were compared against positive (RBC incubated in double-distilled water resulting in total cell haemolysis) and negative (RBC suspended in PBS) controls and are summarised in Figure 5.21.

Results collected with the use of RBC assay correlated with those obtained using the MTT assay. The present study demonstrates that positively charged liposomes are toxic, thus correlating with what has been cited in previous literature (e.g. Lincopan *et al.*, 2006). The toxicity of DSTAP liposomes containing a low level (9-18 μ mol) of positively charged component was found to be especially low. This may be explained by a possible lower extent of association of these liposomes with the respective cells. Biological membranes typically contain a substantial fraction of negatively charged lipids, (Coster and Chilcott, 1996). The negative surface charge renders the membrane sensitive to interactions with cationic moiety. Red blood cells exhibit negative surface charge, which can be attributed to the carboxylic portion of neuraminic acid (Marikovsky and Danon, 1969). At high concentration of charged component here (27 μ mol), however, it seems plausible that a combination of the increased number of charged liposomes and negatively charged membrane may improve chance of contact between the liposome and the cell surface thereby enhancing the association and toxicity (Smistad, 2007).

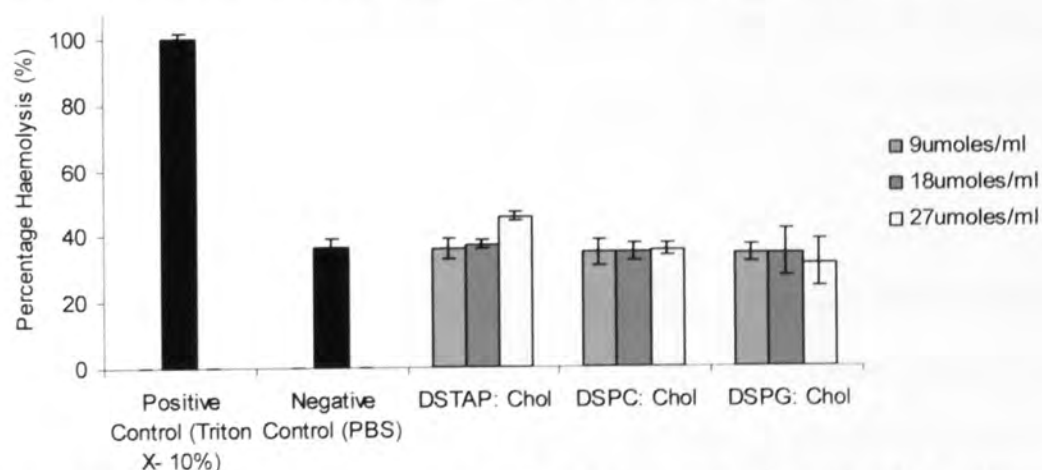


Figure 5.21 Effect of bilayer charge and lipid concentration on the percent haemolysis of red blood cells. Red blood cells (1×10^6 cells/mL) were incubated liposomes (9-27 μ mol/mL) at 37°C for 1 hour. Data was derived from five independently performed experiments and the error bars represent the standard deviation.

5.6 Conclusions

Investigating the effect of liposome composition, encapsulation of midazolam and propofol was shown to be enhanced by adopting longer chain length lipids. However, the converse applied to diazepam suggesting that a range of factors must be considered when formulating lipophilic drugs within bilayers.

Formulations encapsulating midazolam and propofol were successfully prepared from asymmetrical synthetic derivatives of PC. MPSC and POPC formulations provided significantly lower encapsulation rates for the two poorly water-soluble compounds when compared to that of two of their symmetrical counterparts. This suggests that alkyl chain symmetry plays a significant role in the incorporation of hydrophobic drugs which could be accredited to bilayer formation via a mismatch interaction between alkyl side-chains of asymmetric lipids, therefore reducing the overall hydrophobic area for hydrophobic drug interaction, when compared to that of symmetric lipid bilayers. Investigations into combining a symmetrical lipid with an asymmetrical derivative enhanced encapsulation of a hydrophobic drug while reducing that of another suggesting the importance of drug characteristics.

The influence of drug characteristics on bilayer loading was examined. The investigation indicated a greater influence by molecular weight as opposed to lipophilicity of drug in determining encapsulation rates since decreasing molecular weight resulted in enhancing drug loading within liposomes.

Results suggest that a fully charged bilayer plays an influential role in the encapsulation of the poorly water-soluble model drug candidate, propofol. The cationically-charged formulations generally encapsulated significantly more propofol and an anionic formulation yielded significantly lower drug-loading than their neutral counterparts therefore suggesting fully charged bilayer to play a key role in the entrapment of a poorly water-soluble model drug candidate.

Data obtained indicated that the lipid composition of liposomes influenced the released activity of entrapped drug. Drug release was increased by using cationic lipids and lower molecular weight of drug; conversely, a reduction was noted when employing longer chain lipids thus supporting the rational of longer chain lipids producing more stable liposomes, a theory also supported by results obtained via Langmuir studies.

Vesicle sizes for all formulations obtained from TEM images were generally in agreement with the particle size analysis performed on a Malvern Mastersizer. TEM micrographs showed a reduction in aggregation upon the synthesis of a near-fully charged bilayer as opposed to a neutral bilayer.

The effect of various formulation factors on the *in vitro* cellular toxicity of liposomes on COS-7 cells were studied. The factors scrutinised were alkyl chain length of main phospholipid (DSPC and C₂₄PC), phospholipid asymmetry (DSPC vs. MSPC), lipid concentration, and the type of charge in the liposomes. Some results were validated with another *in vitro* protocol, the RBC assay. Several significant main factors and interactions

were revealed. The alkyl chain length of main phospholipids and lipid asymmetry did not influence the toxicity within the experimental set-up. Furthermore, the toxicity of neutral (DSPC) and negatively charged liposomes (DSPG) were absent at all concentrations examined. Positively charged liposomes were shown to be toxic only at a relatively higher concentration of $27\mu\text{mol/mL}$. Results obtained using MTT assay was consistent with those yielded using the RBC assay.

The established relationship between phospholipid tail-length, nature of head-group, lipid symmetry and drug loading could be used in order to be able to predict the characteristics of drug-encapsulated liposomes for those drugs that fall within the range of molecular weights and Log P values investigated here.

Chapter Six:

Fatty alcohols as alternative to cholesterol as liposomal bilayer stabilisers

6.0 Introduction

The potential application of liposomes as therapeutic tools is still confronted by their innate physical and chemical instabilities, which can lead to an enhanced bilayer permeability and eventual loss of drug, vesicle aggregation/fusion, and precipitation (Mohammed *et al.*, 2007). A formulation should ideally display a degree of stability. There are numerous reports of marketed pharmaceutical preparations requiring modifications to improve their stability for administration thus conveying the importance of a stable sample under various conditions. For example, a study (Oussoren *et al.*, 1998) reported that liposomes composed of 'fluid-state' phospholipids only prolonged the damaging effects of doxorubicin when administered subcutaneously and that only with liposomes of a more rigid nature were substantially more effective in preventing local tissue damage over a longer span of time.

In another instance, Zhang *et al.* (2005) had developed a well-characterised novel lyophilised liposome-based paclitaxel preparation that is more stable and sterile proposing it as a viable alternative to the Taxol[®], one of the most effective marketed anticancer drug prescribed for the treatment of breast, ovarian and non-small cell lung cancer. However, its active ingredient, paclitaxel, is only sparingly soluble in water and therefore, intravenous administration depends on the utilisation of the non-ionic surfactant Cremphor[®] EL, which enhances toxicity and results in hypersensitivity reactions in certain patients (Shieh *et al.*, 1997; Zhang *et al.*, 2005), hence the need for an improved version of the preparation.

Although numerous vesicle-forming substances are well recognised, cholesterol is the main membrane-stabilising material extensively investigated (Devaraj *et al.*, 2002). Cholesterol is widely accepted to improve bilayer stability, retard permeation of solutes, prevent leakiness (Devaraj *et al.*, 2002) and broaden and eventually eliminate the cooperative gel-to-liquid phase transition temperature of the phospholipid bilayer (Ohtake *et al.*, 2005). The importance of the compound is well established and in some cases, vesicles do not undergo formation without the presence of it. For example, Gopinath *et al.* (2004) discovered that the thin film of ascorbyl palmitate on hydration did not yield vesicles and only in the presence of cholesterol did the group observe niosome formation. In another example, niosome formation from sorbitan esters (non-ionic surfactants) is also impossible without the inclusion of cholesterol (Uchegbu and Florence, 1995) (more detailed information regarding the compound is mentioned in Section 4.0).

Other than cholesterol, a material with effective liposomal bilayer-stabilising properties is yet to be identified. Devaraj *et al.* (2002) have demonstrated the formation of stable niosomes from fatty alcohols (FA). FA are commonly used in lipid-based drug delivery systems including parenteral emulsions and solid lipid nanoparticles (Dong and Mumper, 2006). They are aliphatic alcohols derived from natural fats and oils, and due to their amphipathic nature, they behave as nonionic surfactants finding use as emulsifiers, emollients and thickeners in cosmetics and food industry. Three FA were investigated initially (tetradecanol, Tet; hexadecanol, Hex; octadecanol, Oct), with one being selected for further scrutiny (properties summarised in Table 6.1).

It is well recognised that the composition of the lipid bilayer, the bilayer-drug interaction, vesicle size, bilayer integrity and storage conditions are all factors influencing liposome stability (du Plessis *et al.*, 1996; Mohammed *et al.*, 2006; Treat *et al.*, 2001). Therefore it was instructive to study and compare the effects of FA and cholesterol on liposomal drug-loading, release rate and stability. Preliminary *in vitro* toxicity studies were also performed.

Propofol was selected as the model drug and incorporated within the liposomes since of all drugs (diazepam, ibuprofen, midazolam, phenytoin, propofol and rifampicin) studied for liposomal encapsulation, formulations encapsulating propofol revealed the highest loading rates (Section 4.1). It is a widely used anaesthetic and is characterised by a water solubility of approximately 153 µg/mL (Altomare *et al.*, 2003), a Log P value of 3.84 and a pKa of 11.1 (Wishart *et al.*, 2006).

As well as acting as model hydrophobic drug for this study, formulations consisting of phospholipids and FA were also investigated as a potential alternative delivery vehicle to emulsion systems.

Fatty alcohol	Synonym	Alkyl chain length of FA (no. of carbon atoms)	Molecular weight	Molecular formula
Tetradecanol	Myristyl alcohol	14	214	CH ₃ (CH ₂) ₁₃ OH
Hexadecanol	Palmitoyl alcohol, Cetyl alcohol	16	242	CH ₃ (CH ₂) ₁₅ OH
Octadecanol	Octadecyl alcohol, Stearyl alcohol	18	270	CH ₃ (CH ₂) ₁₇ OH

Table 6.1 Summary of key features of the fatty alcohols. (Information courtesy of Sigma-Aldrich, 2008)

6.1 The effect of tetradecanol concentration on propofol encapsulation

Examinations using cholesterol in Section 4.1 showed that the correct choice of cholesterol concentration requires to be optimised. On that basis, initial investigations involved determining an appropriate level of FA (tetradecanol, hexadecanol or octadecanol) that will yield the optimum encapsulation of propofol. Tetradecanol was used to achieve an optimum composition. The effects of incorporating the FA within the lipid composition of PC (16 μ mol) liposomes on propofol loading was determined by varying tetradecanol concentration from 0-27 total molar % (Table 6.2).

Initial optimisation studies revealed a suggested trend of decreasing incorporation of propofol with increasing concentration of tetradecanol in the order of PC- only \geq PC: Tet-11% > PC: Tet-20% > PC: Tet-33% (Table 6.2) ($P < 0.05$). This is similar to previous studies (Section 4.1.1) performed in our laboratory where cholesterol-free and formulation constituting 11 total molar % cholesterol encapsulated the highest quantity of various hydrophobic drugs.

Tetradecanol content (total molar %)	Encapsulation (% mol/mol)	Size (μ m)	Zeta potential (mV)
0.0	22.4 \pm 1.2	5.6 \pm 0.2	-3.9 \pm 4.6
11.1	20.8 \pm 0.7	5.2 \pm 0.1	-5.4 \pm 5.2
20.0	19.2 \pm 0.2	4.7 \pm 0.3	-5.1 \pm 4.4
27.3	18.0 \pm 0.2	5.6 \pm 0.2	-1.31 \pm 3.9

Table 6.2 Characteristics of PC liposomes prepared using increasing amounts of tetradecanol (0-27 total molar %) in the presence of 1.0mg of drug. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. The propofol loading of liposomes was analysed as previously described in Section 2.2.3. The drug content was analysed by UV spectroscopy at 268nm wavelength. Results are expressed as the means of at least four experiments \pm S.D.

Unfavourable conditions for propofol-encapsulation at higher portions of tetradecanol could be due to competitive packaging in the bilayer between the FA and the drug

(Mohammed *et al.*, 2004), as may be the case earlier with cholesterol (Section 4.1.1). There could also be the possibility of crystallisation of the FA as a result of exceeding a certain solubility threshold, a phenomenon previously reported with cholesterol (Ohtake *et al.*, 2005). There is an upper limit to the concentration of cholesterol that can be accommodated in a hydrated phospholipid bilayer (ranging from 50 to 66 total molar %); any surplus of the substance will tend to precipitate out, yielding crystals (Ohtake *et al.*, 2005). This theory could also be the reason behind difficulties encountered in hydrating the lipid film containing 33 total molar % tetradecanol as the film could not be effectively removed from the glass surface of evaporating flask, a problem earlier encountered (Section 4.2) when attempting to encapsulate phenytoin in cholesterol containing liposome systems.

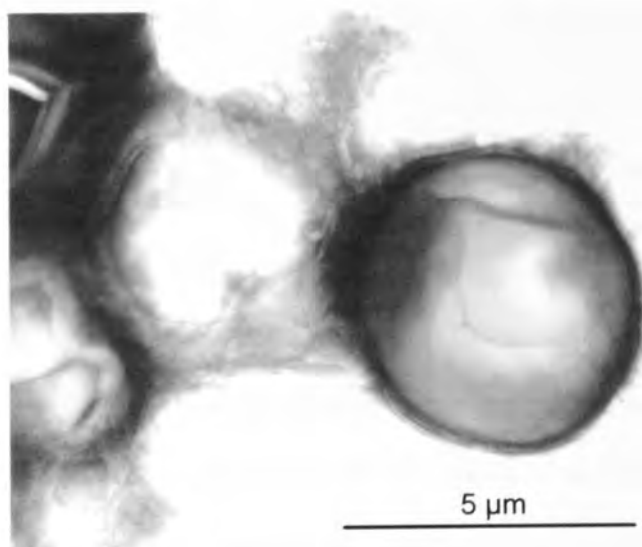


Figure 6.1 A transmission electron micrograph of a propofol-entrapped formulation prepared from 16 μmol PC containing 11 total molar % tetradecanol content. Images were taken using a JEOL 1200EX microscope fitted with a LaB6 filament, with an operating voltage of 40-120kV.

In terms of vesicle size and surface charge no significant difference in vesicle size was observed with the incorporation of propofol or increasing tetradecanol content. Moreover, TEM analysis (Figure 6.1) revealed spherical and homogenous vesicles that were in agreement with particle size analysis (Table 6.2). Concentration of tetradecanol did not influence the size or appearance of vesicles viewed on images obtained using this instrument (results not shown). Similarly, zeta potential was unaffected by the presence or absence of tetradecanol (Table 6.2).

A FA concentration of 11 total molar % was implemented for all further formulations to investigate if incorporation of FA could enhance stability as has been previously reported with cholesterol liposomes (Drummond *et al.*, 1999; Gregoriadis and Senior, 1980; Zhang *et al.*, 2005) that a formulation prepared with stabiliser is more stable than without.

6.2 Investigating alkyl chain length of fatty alcohol

To investigate if length of FA alkyl side moiety influenced solubilisation of drug, tetradecanol and two of its analogous structures, hexadecanol and octadecanol, were studied in PC formulation, each formulation containing 11 total molar % of the saturated aliphatic alcohol. Each formulation was examined with respect to encapsulation, stability and release and compared against the corresponding results obtained earlier for cholesterol (Sections 4.1.1, 5.1.4 and 5.1.3, respectively).

6.2.1 The effect of fatty alcohol alkyl chain length on propofol-loading

The effect of alkyl chain length of FA on drug solubilisation was investigated using liposomes formulated from PC and tetradecanol or hexadecanol or octadecanol and compared to previous results collected for sample containing cholesterol (Section 4.1.1).

Investigations of liposomes containing the various FA tested revealed similar levels of propofol encapsulation, ranging between ~19 and ~21 % mol/mol. There is a suggestion of a trend in decreasing loading with increasing FA alkyl chain length however this was not significantly different, and none of the FA formulations had significantly lower drug loading than their cholesterol containing counterparts (22 % mol/mol) (Table 6.3).

This is an improvement on the data obtained for niosomes (Devaraj *et al.*, 2002) where the vesicles containing the same FA displayed a slightly lower loading capacity when compared against those containing cholesterol (65.5, 64.0, 63.5% entrapment for tetradecanol, hexadecanol and octadecanol, respectively, versus 70.3% entrapment for cholesterol).

Neither the hydrocarbon chain length of FA nor the inclusion of cholesterol yielded any significant change in the size of the liposomes, correlating with results published by Devaraj *et al.* (2002). Likewise, zeta potential did not show any relation with alkyl chain length (Table 6.3), an observation also seen with the niosomal preparations reported by Devaraj *et al.* (2002).

Formulation (0.89: 0.11 molar ratio)	Alkyl chain length of FA (no. of carbon atoms)	Encapsulation (% mol/mol)	Size (μm)	Zeta potential (mV)
PC: cholesterol	-	22.0 ± 0.4	6.6 ± 0.5	-5.1 ± 0.9
PC: Tet	12	20.8 ± 0.7	5.2 ± 0.1	-5.4 ± 5.2
PC: Hex	16	19.7 ± 1.4	6.1 ± 0.6	3.2 ± 4.9
PC: Oct	18	18.9 ± 2.6	5.1 ± 0.2	4.1 ± 3.1

Table 6.3 The influence of alkyl chain length of fatty alcohol on propofol entrapment, vesicle size and surface charge with comparisons against cholesterol-based preparation. Liposomes encapsulating propofol were prepared from 16 μmol PC and 11 total molar % fatty alcohol or cholesterol in the presence of 1.0mg of drug. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. The propofol loading of liposomes was analysed as previously described in Section 2.2.3. The drug content was analysed by UV spectroscopy at 268nm wavelength. Results are expressed as the means of at least four experiments \pm S.D. (Data for cholesterol formulation was previously presented in Section 4.1, is presented here for ease of comparison)

6.2.2 Stability studies

The objectives of this study were to determine the effect of bilayer composition on the physical stability of the vesicles during a 28day storage period at 4°C and 25°C. Stability studies of liposomes loaded with propofol at 4°C and 25°C with respect to retention of the entrapped drug and changes in the size distribution were followed. Five PC formulations were prepared: three containing 11 total molar % tetradecanol, hexadecanol or octadecanol each; the fourth preparation containing 11 total molar % cholesterol, with all comparisons made against PC-only liposome.

No significant difference in mean volume distribution between all five MLV formulations was noted, with vesicle sizes in the range of 5-6 μm (Figure 6.2). Further for all formulations, these vesicle sizes remained constant for up to 28day at either 4°C or 25°C (Figures 6.2a and 6.2b, respectively).

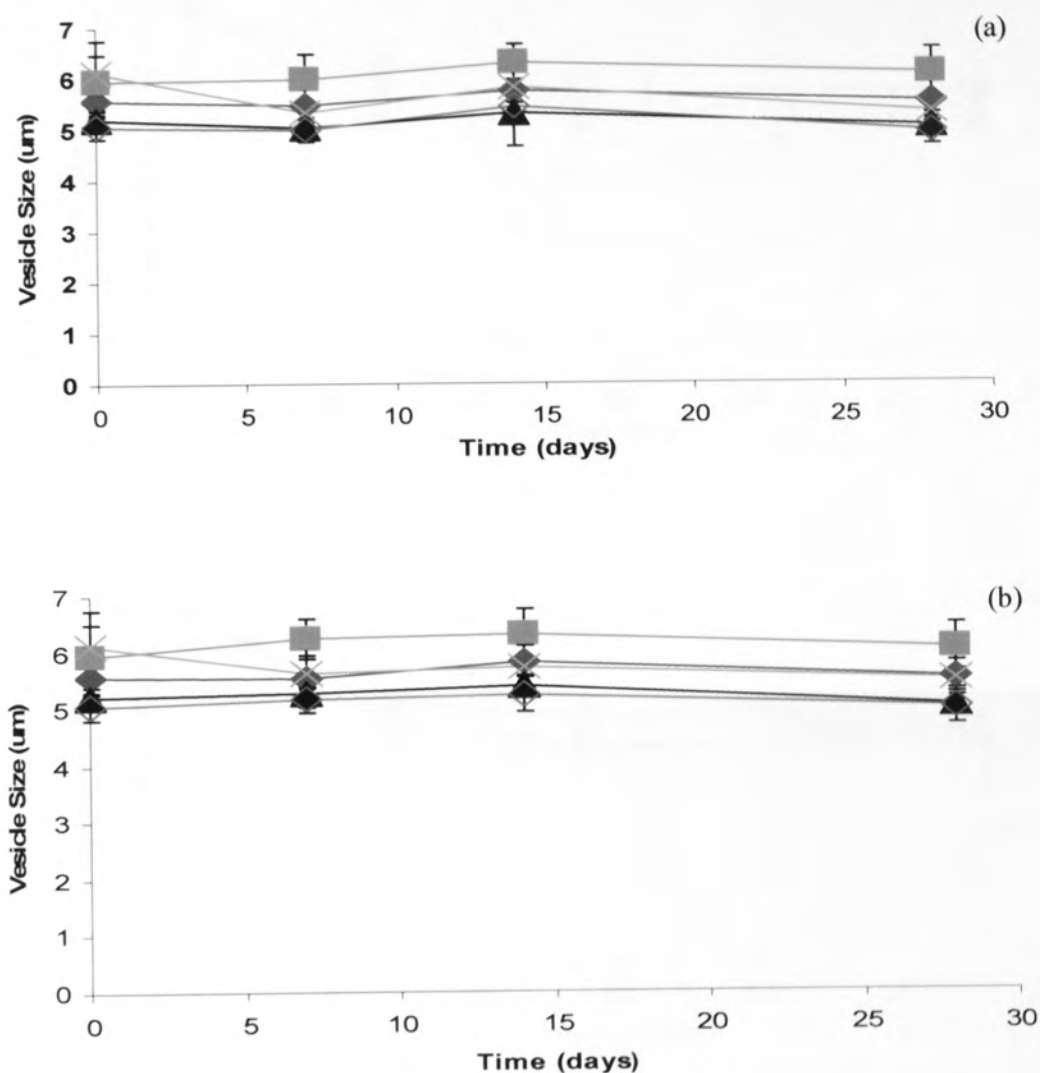


Figure 6.2 Vesicle size of PC (◆), PC: cholesterol (■), PC: Tet (▲), PC: Hex (x) and PC: Oct (◇) formulations encapsulating propofol, stored at 4°C (a) and 25°C (b), for 28day. Mean volume diameter of vesicles were determined as described in Section 2.2.8 Results are expressed as the means of four experiments \pm S.D. (Data for cholesterol formulation was previously presented in Section 5.1.4, and is presented here for ease of comparison)

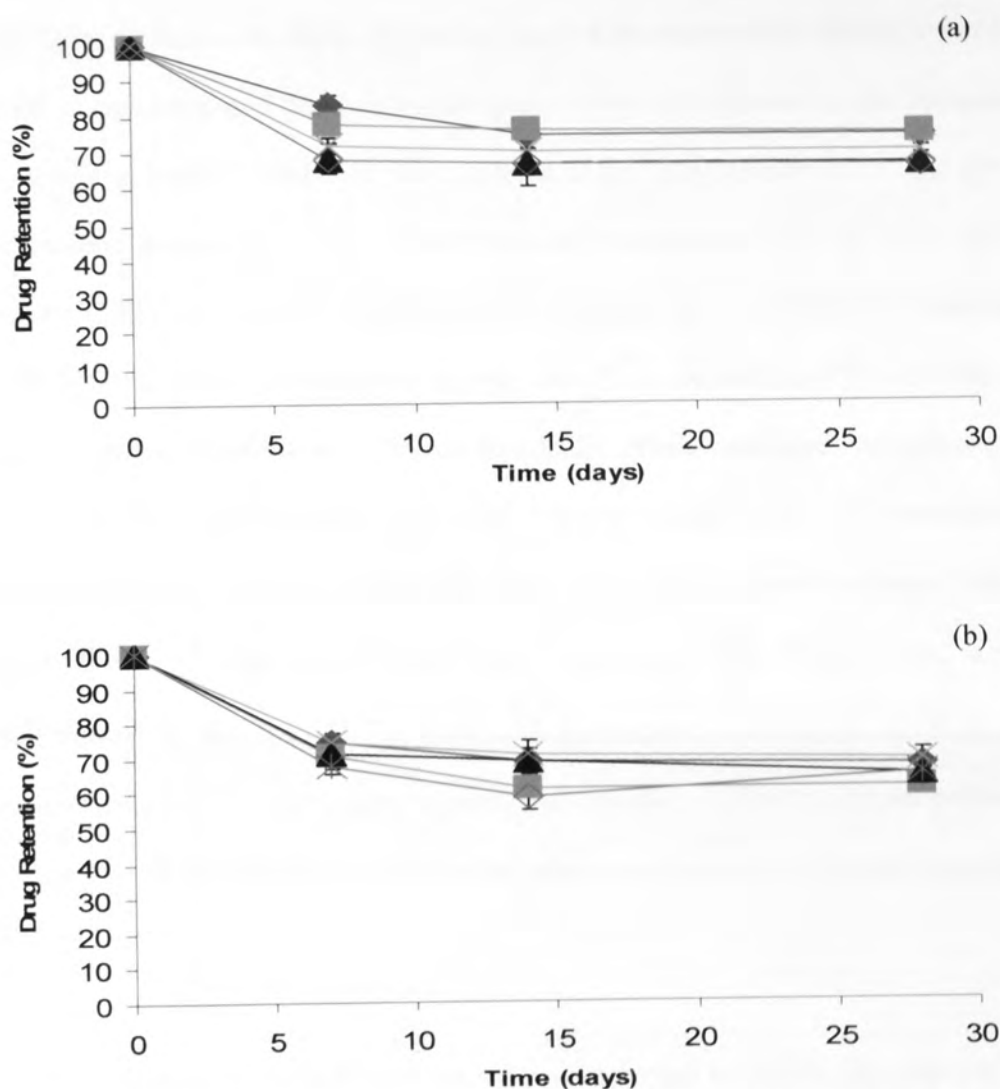


Figure 6.3 Propofol retention of PC (♦), PC: cholesterol (■), PC: Tet (▲), PC: Hex (x) and PC: Oct (◇) formulations in aqueous buffer, pH = 7.4, stored at 4°C (a) and 25°C (b), for 28day. Propofol was encapsulated and drug retention was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27,200g for 30min. Results represent percentage retention of initially incorporated propofol. Results are expressed as the means of four experiments \pm S.D. (Data for cholesterol formulation was previously presented in Section 5.1.4, is presented here for ease of comparison)

Propofol retention profiles were alike for both FA and cholesterol formulations at 4°C and 25°C for periods up to 28day (Figures 6.3a and 6.3b, respectively). Following initial removal of non-entrapped drug via centrifugation, propofol retention to the liposomes after storage at both 4°C and 25°C was analysed. After 7day storage at 4°C and 25°C, drug retention dropped to around 70-80% for all formulations stored at 25°C. When stored at 4°C all three FA containing formulations samples also lost 20-30% of their drug load, however liposomes composed of PC-only and PC: cholesterol, at 4°C, were able to retain more than 80% of the encapsulated drug under similar conditions. At time points thereafter, despite a slight leakage in general, retention values for all five preparations remained constant with no further significant loss of propofol being detected up to 28day; between 70-80% of drug was retained for all formulations after 28day at 4°C and a slightly reduced 65-70% at 25°C. The initial drop in drug retention could be as a result of unfavourable molecular arrangement of some hydrophobic propofol molecules between the hydrophilic phospholipid head-group in the bilayer as discussed previously in Section 5.1.4

In summary, the mean volume distribution and retention rates for liposomes composed of PC only were not significantly different from preparations containing cholesterol or a FA at either storage conditions up to 28day.

6.2.3 Effect of alkyl chain length of stabiliser on drug release

Devaraj *et al.* (2002) showed that while niosomes containing the FA exhibited a release pattern similar to that of the cholesterol formulation, the release rates of the FA samples

were in fact slower than that of cholesterol. Furthermore, the group observed a general increase in release rate with decreasing alkyl chain length of the polyoxyethylene sorbitan monoester surfactant. The effect of the particular FA on the release of propofol was examined by preparing liposomes using the alcohol series and compared to formulations containing with and without cholesterol. Propofol release from the five formulations (PC-only, PC: cholesterol, PC: tetradecanol, PC: hexadecanol and PC: octadecanol) was studied based on drug release in physiological buffer, pH = 7.4, as release medium at 37°C (up to 72hour).

Cumulative percentage propofol release versus time plots of liposomes are displayed in Figure 6.4. The release of propofol was the slowest from the formulation containing tetradecanol and the fastest from the vesicles absent of either cholesterol or a fatty alcohol. Initially the cumulative release rate of propofol from the various formulations was not significantly different with 20-25% of propofol released after 12hour. However after 24hour, differences in the release profile were more apparent with PC-only liposome showing higher release rate compared to the other formulations. A similar pattern was noted at 48hour. After 72hour, cumulative release rate decreased in the order of PC: Tet > PC-only = PC: Hex > PC: Oct > PC: cholesterol ($P < 0.05$, at 72hour).

These data suggests that the length of the FA alkyl chain length plays a key role in bilayer permeability and influences liposomally incorporated drug release. Formulations containing octadecanol within their composition released propofol at a significantly lower rate when contrasted with formulations including tetradecanol or hexadecanol ($P < 0.05$). However all FA formulations displayed higher release than the formulation containing

cholesterol therefore indicating FA to be less effective than cholesterol in retaining propofol. After 72hour, only liposomes containing octadecanol or cholesterol gave a slower release profiles than the PC-only formulation.

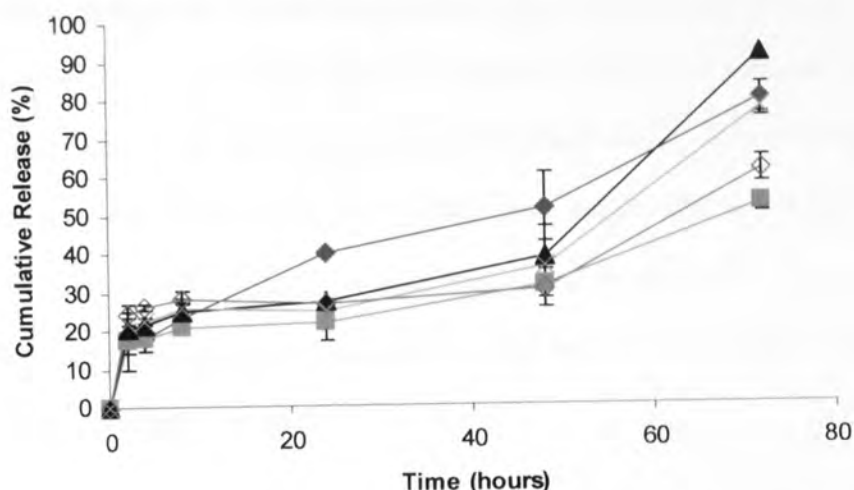


Figure 6.4 The effect of fatty alcohol alkyl chain length on drug release. Propofol release under physiological conditions from PC (♦), PC: cholesterol (■), PC: Tet (▲), PC: Hex (×) and PC: Oct (○) formulations in aqueous buffer, pH = 7.4, at 37°C. Propofol was encapsulated and drug release was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27, 200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments \pm S.D. (Data for cholesterol formulation was previously presented in Section 5.1.3, is presented here for ease of comparison)

The generally reduced permeability of stabiliser-containing bilayers compared to cholesterol-free membranes is probably due to space-filling action as a result of accommodation of stabiliser in the molecular cavities by construction of phospholipids into liposomes, as reported previously (Devaraj *et al.*, 2002) for cholesterol. Bilayer drug release is recognised to be influenced by the hydrophobicity of the lipid excipients within the vesicles (Mohammed *et al.*, 2004; Yoshioka and Florence, 1994). Hydrophobicity is significantly influenced by the alkyl hydrocarbon chain length of a compound (Yoshioka

and Florence, 1994), which could explain the slower drug-release rate for octadecanol formulation when weighed against tetradecanol and hexadecanol formulations.

6.3 The effect of lipid alkyl chain length used in combination with fatty alcohols

The effect of the phospholipid alkyl chain length used in combination with a FA (tetradecanol) on bilayer drug loading (propofol) and release was also investigated. Liposomes prepared from PC (average C₁₂ alkyl chain length) and its derivatives, DMPC (C₁₄ alkyl chain length), DPPC (C₁₆ alkyl chain length) and DSPC (C₁₈ alkyl chain length) were prepared in combination with 11 total molar % tetradecanol concentration, as decided by optimisation studies (Section 6.1).

6.3.1 Effect on drug loading

Encapsulation studies using PC and its derivatives (Figure 6.5) suggest that the longer chain DSPC entraps the highest quantity of propofol when weighed against its shorter chain counterparts, DPPC, DMPC and PC, where a trend of increasing propofol incorporation with increasing lipid alkyl chain length in the order of DSPC > DMPC = DPPC > PC was observed with incorporation values ranging from ~20 to 25 % mol/mol.

The trend obtained correlates with a previous study (Section 5.1.1) investigating these phospholipids in combination with cholesterol and is also consistent with previously cited work investigating the role of alkyl chain length (Gregoriadis, 1973; Manosroi *et al.*, 2003; Mohammed *et al.*, 2004). This suggests that the choice of phospholipid used in the preparation of liposomes influences the bilayer loading of a drug independent on the

choice if bilayer stabiliser (cholesterol or fatty alcohol) used. As previously mentioned (Section 5.1.1), this could be related to the increased hydrophobic volume and/or hydrophobic bonding offered by the longer alkyl chain lipids.

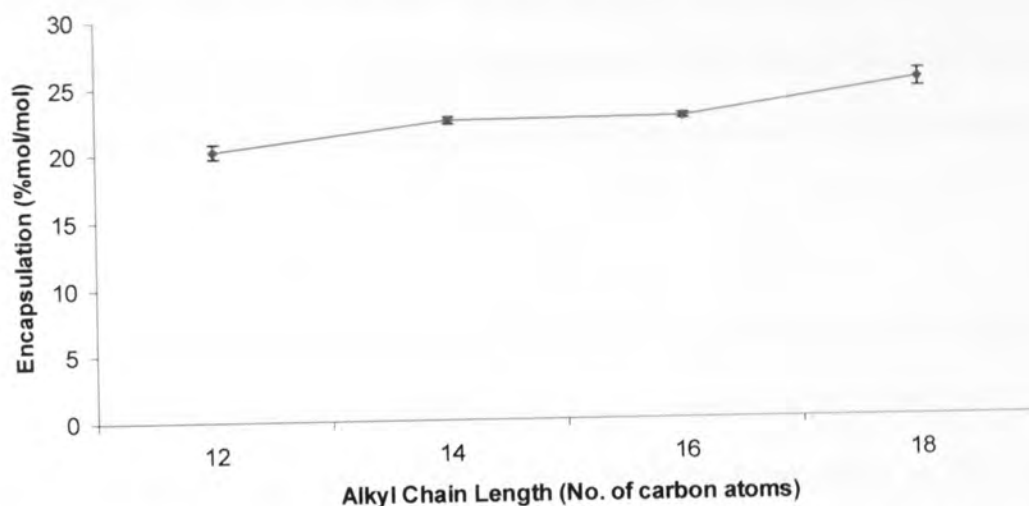


Figure 6.5 The influence of lipid alkyl chain-length in combination with tetradecanol on drug encapsulation. The drug loading of vesicles was determined as stated in Section 2.2.3. The propofol content was analysed by UV spectroscopy at 268nm wavelength. Results are expressed as the means of four experiments \pm S.D. (Data for PC: Tet formulation was previously presented in Section 6.2.1, is presented here for ease of comparison)

Formulation (0.89: 0.11 molar ratio)	Size (μ m)	Zeta potential (mV)
PC: Tet: Prop	5.2 \pm 0.1	-5.4 \pm 5.2
DMPC: Tet: Prop	7.2 \pm 0.4	7.3 \pm 5.3
DPPC: Tet: Prop	8.7 \pm 0.5	-4.6 \pm 3.5
DSPC: Tet: Prop	9.2 \pm 0.5	2.1 \pm 3.8

Table 6.4 The influence of lipid alkyl chain-length in combination with tetradecanol on vesicle size and surface charge. The mean volume diameter of vesicles and zeta potential were determined as described in Section 2.2.8 and Section 2.2.9, respectively. Results are expressed as the means of four experiments \pm S.D. (Data for PC: Tet formulation was previously presented in Section 6.2.1, is presented here for ease of comparison)

Liposomes formulated with DMPC, DPPC or DSPC in combination with tetradecanol were all significantly larger ($P < 0.05$) in size than their PC liposome counterparts (Table

6.4) with a trend of increasing vesicle size with increasing lipid alkyl chain length. This increase in mean volume diameter could be attributed to longer alkyl-chain groups inducing a physical expansion of the hydrophobic bilayer as drug-free vesicles revealed similar results (not shown) thereby eliminating any potential effect of increased drug loading on vesicle size. Zeta potential did not show any significant difference with alkyl chain length of phospholipids with all formulations ranging around neutrality (Table 6.4).

6.3.2 *Effect on drug release*

To investigate the role of the alkyl chain length on the drug release rate offered during *in vivo* temperature, propofol-encapsulated liposomes prepared from PC, DMPC, DPPC and DSPC (stabilised with addition of 11 total molar % tetradecanol) at 37°C were investigated (Figure 6.6).

Investigating formulations containing tetradecanol, the results demonstrate that significant changes in release rates were observed with changes in alkyl chain length of phospholipid. The cumulative release rate generally increased with a decrease in the chain length of phospholipids in the order of $PC \geq DMPC > DPPC > DSPC$ at 72hour ($P < 0.05$, at 72hour) (Figure 6.6). Both DSPC and DPPC formulations showed sustained release of propofol (45% and 65% of the total entrapped, respectively) after 72hour. This is in comparison to PC and DMPC formulations, whilst initially (up to 48hour) showing a sustained release profile, rose to about 90% after 72hour.

In general, over a 72hour period, all tetradecanol-based formulations appeared to enhance drug permeability when weighed against an equivalent concentration of cholesterol. This

is interesting since there was no significant difference in drug loading between formulations (of equivalent phospholipid alkyl chain length) containing the two substances (Section 5.1.1 vs. Section 6.3.1). This could be attributed to variations, with respect to cholesterol molecules, in how tetradecanol molecules are arranged amongst the alkyl chains of phospholipids leading to lower levels of bilayer rigidity. It is to be noted that perhaps octadecanol would be comparable with cholesterol in release studies but gave less entrapment therefore overall cholesterol is still a more effective bilayer stabiliser.

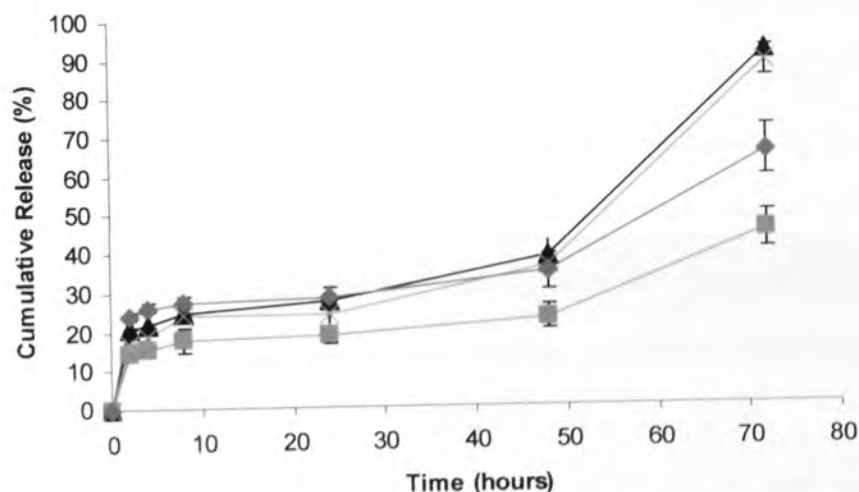


Figure 6.6 The influence of lipid alkyl chain-length in combination with tetradecanol on drug release. Propofol release under physiological conditions from PC: Tet (▲) DMPC: Tet (x), DPPC-Tet (◆), DSPC-Tet (■), formulations (0.89: 0.11 molar ratio) in aqueous buffer, pH = 7.4, at 37°C. Propofol was encapsulated and drug release was measured on the basis of untrapped propofol recovered in the suspension following centrifugation at 27, 200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated propofol and are expressed as the means of four experiments \pm S.D. (Data for PC: Tet formulation was previously presented in Section 6.2.3, is presented here for ease of comparison)

6.4 Influence of drug properties on release

It is understood that drug molecules might significantly adjust basic features of vesicles such as membrane integrity (Fatouros and Antimisiaris, 2002). It is also stated that the rate of release from liposomal preparations is drug-dependent; for example, doxorubicin is released slowly from vesicles while vincristine leaks out rapidly (Yamouchi *et al.*, 2007). Interestingly, it was shown earlier with liposomes containing cholesterol that membrane encapsulation of drug is dictated by MW which appeared to be directly proportional to encapsulation rates (Section 5.4).

A key point in study of liposomal drug delivery systems is to evaluate the rate at which the drug is released from the liposomal carrier systems since the greatest improvements in therapeutic benefits are attained if the drug is retained in the carriers for several hours following administration (Yamauchi *et al.*, 2007). The aim of this study was therefore to identify possible association between drug encapsulation and release from liposomes as well as any connection between various characteristics of drugs and bilayer release.

Using the FA-based liposomal systems, the study of release of drug from similar membranes was performed with three drugs that have varying molecular size and pKa values, but similar Log P values. Vesicles containing a consistent quantity of tetradecanol (11 total molar %) were prepared, encapsulating midazolam, propofol or rifampicin (summary of drug characteristics presented in Table 6.5). Drug release from the three formulations was investigated based on release drug in physiological buffer, pH = 7.4, as release medium at 37°C (up to 72hour).

Results presented in Figure 6.7 demonstrate that loss of drug from the liposomal membrane is affected by the drug characteristics. Formulations incorporating midazolam or rifampicin appear to resemble a sustained-release profile up to 72hour, with a cumulative release of approximately 40% and 20%, respectively, at completion of study. Whilst in comparison the propofol-loaded preparation, although drug is released in sustained fashion for up to 48hour, a subsequent 'burst'-release about 50% of initial entrapped drug occurs during the final 24hour, with a near-complete release by end of study period.

Drug	Molecular weight (Da)	Log P	Dissociation constant (pKa)	Solubility (µg/mL)	Overall nature of drug (functional group)
Midazolam	326	3.87	6.2 (Monzon and Yudi, 2001)	240	Basic (Andersin and Tammilehto, 1995)
Propofol	178	3.84	11.1	153 (Altomare <i>et al.</i> , 2003)	Acidic
Rifampicin	833	3.72	1.7 & 6.8 (Pasquardini <i>et al.</i> , 2008)	Unknown	Acidic (Ovcharova <i>et al.</i> , 1990)

Table 6.5 Summary of the characteristics of all model drug candidates investigated. Data obtained from Wishart *et al.* (2006) unless otherwise stated. (Data previously presented in Section 3.0)

Release data collected (Figure 6.7) present an interesting connection with molecular weight of drug and encapsulation previously observed in Section 5.4. Encapsulation (albeit in formulations stabilised with cholesterol) and cumulative release was presented in the order of propofol > midazolam > rifampicin, reflecting their decreasing MW. Therefore it is very possible that MW plays an influential role in dictating levels of drug encapsulation as well as the release. An investigation by Maurer *et al.* (2001) describes the interaction of nucleotide-based polyelectrolytes with cationic liposomes. Leakage of

calcein, a small molecule with a MW of 623Da, leaked at a significantly faster rate than FITC-dextran, a much larger compound (MW of 19,500Da), thereby suggesting bilayer leakage is MW-dependent.

There did not appear to be any other correlations with the other properties of the three drugs such as solubility in aqueous media and dissociation constant (results not shown).

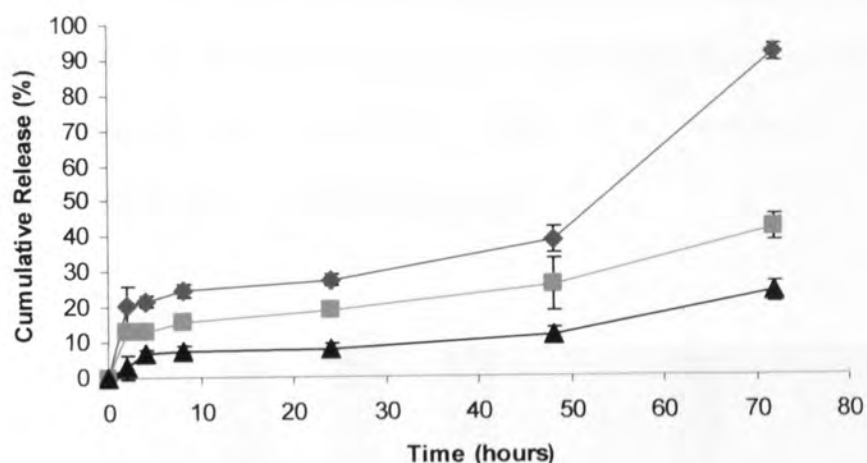


Figure 6.7 The effect of drug on the release profile of either midazolam (■), propofol (◆) or rifampicin (▲) from PC: tetradecanol formulation (0.89: 0.11 molar ratio), in aqueous buffer, under physiological conditions at 37°C. Drug was encapsulated and its release was measured on the basis of untrapped drug recovered in the suspension following centrifugation at 27,200g for 30min at each time-point. Results represent percentage cumulative release of initially incorporated drug and are expressed as the means of four experiments \pm S.D. (Data for PC: Tet formulation was previously presented in Section 6.2.3 and Section 6.3.2, is presented here for ease of comparison)

6.5 Cell toxicity of liposomes containing either cholesterol or tetradecanol

A decrease in cellular metabolic activity is a good marker of early cell damage (Chen *et al.*, 2005). The reduction of cellular metabolic activity of COS-7 cells (African green monkey kidney fibroblast cells), after exposure to liposomes, was determined *in vitro* by

MTT assay at pH = 7.4 (Chen *et al.*, 2005). A DSPC formulation containing 11 total molar % cholesterol was compared to an analogous preparation containing tetradecanol, with respect to cell toxicity via detection of the quantity of formazan produced.

The results of the MTT assay are shown in Figure 6.8. For 4hour incubation with MLV preparations, recorded cellular activity suggests that DSPC: cholesterol and DSPC: tetradecanol formulations were both well tolerated by the COS-7 cells. The number of viable cells remained above 90%; therefore no statistically significant reduction in activity was observed when 18 μ mol/mL of either DSPC: tetradecanol or DSPC: cholesterol was incubated with COS-7 cells at 37°C.

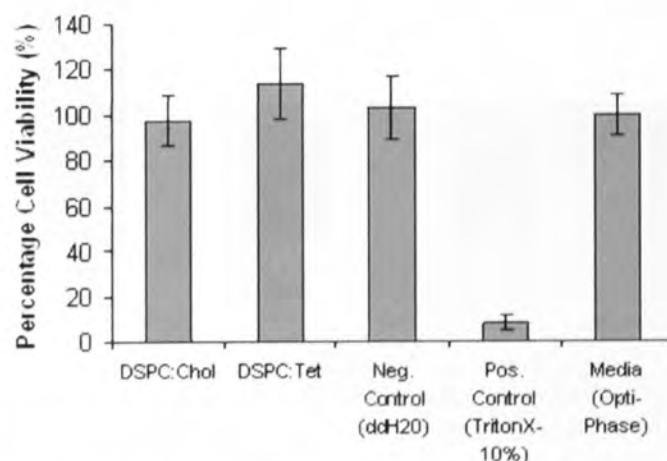


Figure 6.8 Mitochondrial dehydrogenase activity of the COS-7 cells determined by means of the MTT assay after 4hour incubation with liposomes, containing cholesterol or tetradecanol, at 37°C. Concentration of phospholipids introduced into test system was 18 μ mol/mL. Results are expressed in percentage of viable cells with respect to standardised control (medium and cells) and set as mean and standard deviation, $n = 5$.

Results attained by means of the MTT assay (Figure 6.8) were verified using the RBC assay. The haemolysis method scrutinises the membrane-disruptive action of vesicles by way of photometric detection of oxyhaemoglobin following RBC lysis (Chen *et al.*, 2005;

ECVAM, 1992). The two formulations examined above (DSPC: tetradecanol and DSPC: cholesterol) were prepared in physiological conditions ($\text{pH} = 7.4$) and incubated with an appropriate quantity of RBC suspension, centrifuged and absorbance of supernatant analysed. Results were compared against positive (RBC incubated with 10% triton-X resulting in total cell haemolysis) and negative (RBC suspended in PBS) controls and are summarised in Figure 6.9. Outcome of investigation revealed no significant variance in initiation of RBC lysis from that of negative control, with cell damage standing at 30% relative to that of positive control.

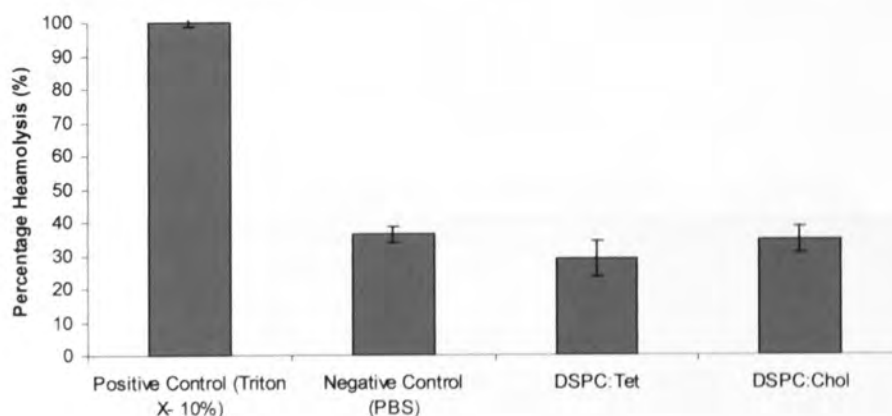


Figure 6.9 Effect of liposomes, with inclusion of tetradecanol or cholesterol, on the percent haemolysis of red blood cells. Red blood cells (1×10^6 cells/mL) were incubated with liposomes ($18 \mu\text{mol/mL}$) at 37°C for 1 hour. Data was derived in triplicate and the error bars represent the standard deviation.

These preliminary toxicity studies suggest that tetradecanol, pertaining to the FA group of compounds, as expected, potentially poses no significant risk (or risk exceeding that of cholesterol's) to cellular bodies at the concentration considered ($18 \mu\text{mol/mL}$). However, additional toxicity tests may be required to further substantiate the claim for tetradecanol and to rule out any potential toxicity induced by the analogues of the FA.

6.6 Langmuir monolayer investigations

Above studies revealed that liposomes could successfully be formulated with DSPC using 11 total molar % tetradecanol as a bilayer stabiliser. Various features were examined with comparisons made against DSPC: cholesterol formulation. Results obtained revealed that both formulations share similar vesicle size, drug encapsulation, surface charge and drug retention after 28day, although the tetradecanol preparation appeared to release considerably more propofol after 72hour when incubated at physiological temperatures compared to its counterpart (summary in Table 6.6). It was clear that further studies were required to examine at molecular level, how each substance behaved in the bilayer.

Parameter	DSPC: Tet	DSPC: Chol
Encapsulation (% mol/mol)	25.1 \pm 0.7	24.8 \pm 1.5
Size (μ m)	9.2 \pm 0.5	8.5 \pm 1.1
Zeta potential (mV)	2.1 \pm 3.8	-4.4 \pm 3.7
Cumulative release after 72hour (%)	45.0 \pm 4.7	33.5 \pm 2.0
Drug retention after 28day at 4°C (%)	74.5 \pm 4.8	76.5 \pm 4.8

Table 6.6 Summary of results comparing DSPC formulations containing tetradecanol and cholesterol with respect to various parameters. Results are expressed as the means of at least four experiments \pm S.D.

Langmuir monolayer studies were used to investigate the interactions at the air/water interface between DSPC and tetradecanol and compared with equivalent cholesterol-based formulation. Mixtures (and the one-component films) of DSPC and tetradecanol or cholesterol at molar fraction 0.11 and their surface pressure- mean molecular area (Π -A) isotherms are reported in Figure 6.10, with calculations presented in Table 6.7. The Π -A isotherm for pure DSPC was in agreement with results collated by Cardenas *et al.* (2005)

with a molecular area calculated as $51\text{\AA}^2/\text{molecule}$. For cholesterol, the swift, practically linear, increase of surface pressure up to point of collapse beyond $40\text{\AA}^2/\text{molecule}$ indicates a closely-packed monolayer structure that exists as a solid-phase during the compression with mean molecular area avoiding radical alterations during the process, as similarly recently reported by Lancelot *et al.* (2007).

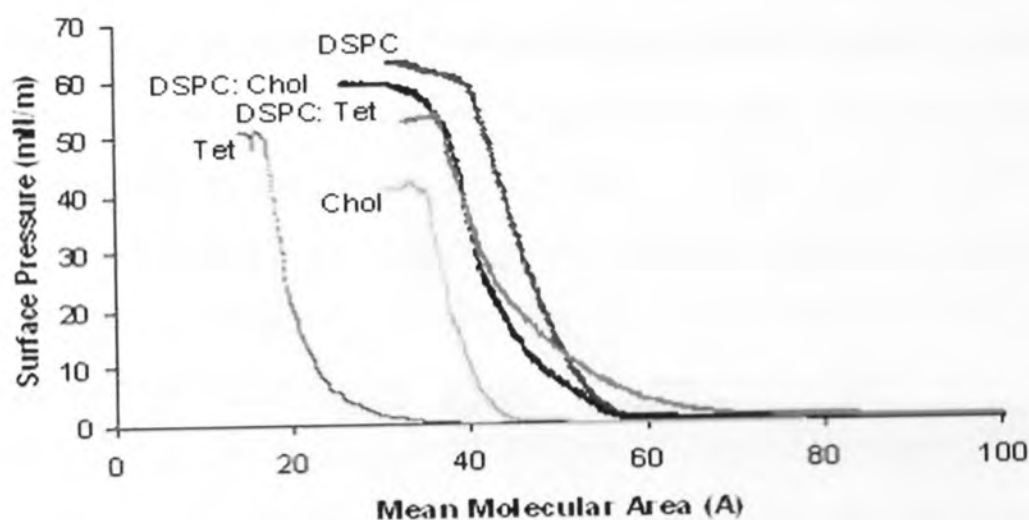


Figure 6.10 The surface pressure-area isotherms of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC, tetradecanol and cholesterol. Results are expressed as the means of three experiments \pm S.D.

Formulation	Extrapolated area at zero pressure ($\text{\AA}^2/\text{molecule}$)	Ideal extrapolated area at zero pressure ($\text{\AA}^2/\text{molecule}$)	Deviation from ideality (%)	Collapse pressure (mN/m)
DSPC	51.0	-	-	63.2
Cholesterol	39.5	-	-	44.2
Tetradecanol	21.0	-	-	51.8
DSPC: cholesterol -11%	45.3	49.7	-8.9	59.3
DSPC: tetradecanol -11%	45.2	47.7	-5.2	54.0

Table 6.7 The experimental and ideal extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface (at 20°C) by DSPC, cholesterol and tetradecanol. Results are expressed as the means of three experiments.

In the case of surfactant mixtures, all isotherms were found to lie between the ranges of those of the pure components (Figure 6.10). The effective area per molecule for the mixtures deviated negatively from the additivity rule (Hac-Wydro and Wydro, 2007) (Table 6.7), suggesting a 'condensing effect' by both tetradecanol and cholesterol. The 'condensing effect' of cholesterol has been presented in the past (Devaraj *et al.*, 2002), which was attributed to the accommodation of the stabiliser in the molecular cavities generated via the assembling of surfactant monomers into vesicles. However, the results obtained show that effective mean area per molecule for DSPC: cholesterol mixture deviated nearly 3% more from ideality than the FA formulation (-8.4% vs. -5.6%, respectively) (Table 6.7). This evidence combined with cholesterol being a much more bulky structure indicates that the condensing influence by tetradecanol is less than compared with cholesterol. This enhanced intercalation of the DSPC: cholesterol compared with DSPC: tetradecanol could explain why the tetradecanol formulation released more than 11% drug compared to cholesterol formulation after 72hour (Table 6.6) with the stronger complexing reducing bilayer permeability (Demel *et al.*, 1972; Mohammed *et al.*, 2004).

6.7 Conclusions

A key predicament in the pharmaceutical application of liposomes is their efficient and stable encapsulation with drugs. Phosphatidylcholine liposomes could successfully be prepared from fatty alcohols therefore proposing as an alternative stabiliser to cholesterol. Vesicles containing tetradecanol, hexadecanol or octadecanol were manufactured and compared to formulations with or without cholesterol with respect to encapsulation

efficiency of the hydrophobic propofol, vesicle size, stability, drug release profile, and toxicity.

Vesicle formation was visually confirmed via viewing of the liposomal suspension using an optical microscope. The vesicles were spherical structures and the mean volume distribution was in the 5-9 μ m range (as confirmed by size measurements using laser diffraction).

The retention of encapsulated drug and size of vesicle were used as a measure of membrane integrity of propofol-loaded vesicles. The results of these preliminary stability investigations revealed generally common characteristics with respect to size and drug retention profiles between the fatty alcohol formulations and the cholesterol formulation. No significant change in average vesicle size was observed between the fatty alcohol and cholesterol preparations for up to 28day at either 4°C or 25°C. Despite a slight enhanced leakage of drug in general at 25°C when compared to 4°C, retention values for preparations containing a fatty alcohol or \pm cholesterol remained constant (up to 80%) with no further significant losses of propofol detected up to 28day at both storage conditions.

Liposomal encapsulation of propofol did not exhibit any relationship with alkyl chain length of fatty alcohol. Generally, encapsulation of propofol was enhanced by employing longer-chain phospholipids (therefore higher transition temperature lipids), although

drug-loading per mmol is constant, regardless of whether liposomes were prepared from fatty alcohols or cholesterol.

The release profiles of liposomes revealed that the presence of fatty alcohols in vesicles stabilises the bilayers and reduces their permeability compared to their absence in liposomes. Results obtained from several release studies overwhelmingly conclude that the length of alkyl chain length (which correlate with transition temperature) of phospholipids play an essential role in bilayer permeability to a hydrophobic drug. Generally, permeabilisation of lipid membranes was dramatically enhanced upon increasing alkyl chain length, and therefore increasing hydrophobic region.

Cell toxicity of DSPC liposomes prepared from either cholesterol or tetradecanol was evaluated by the MTT and RBC assays. Cell integrity was not significantly affected by the presence of 18 μ mol of either compound, although further toxicity tests will be required to further substantiate the conclusion.

The general similarities in the characterisation of liposomes containing FA and cholesterol suggest a common behavioural influence for both compounds within the bilayer. Furthermore, following Langmuir monolayer studies for both DSPC: cholesterol and DSPC: tetradecanol mixtures, the measured mean molecular areas for mixed surfactant monolayers were smaller than the predicted ones for the ideal mixtures, thereby suggesting a strong interaction between DSPC and 11 total molar % cholesterol or tetradecanol. However, evidence obtained suggests that tetradecanol may have a

reduced influence within the bilayer compared to cholesterol, manifesting as relatively decreased packing densities of phospholipid molecules by the former. This would suggest the cholesterol remains the main lipid of choice to be used in bilayer stabilisation although it may be possible to stabilise liposomes with alternatives to cholesterol.

Chapter Seven:

Investigating liposomes using ESEM

7.0 Introduction

Techniques such as scanning electron microscopy (SEM) (Lo *et al.*, 2004), transmission electron microscopy (TEM) (Vangala *et al.*, 2006), cryo-TEM (Ickenstein *et al.*, 2006) and freeze-fracture electron microscopy (Csiszar *et al.*, 2003) have been used extensively to study the characteristics of liposomes. However, the majority of these procedures require preparations to be dried, fixed, frozen or coated with conducting substances prior to imaging therefore not representing an accurate picture of these vehicles in real-time conditions (Perrie *et al.*, 2007; Vangala *et al.* 2006). Environmental scanning electron microscopy (ESEM) is a new technique for studying MLV (Mohammed *et al.*, 2004; Mohammed *et al.*, 2007) that can generate images of wet systems without the requirement of pre-preparation, therefore not structurally or chemically altered, under a range of pressures and temperatures (Vangala *et al.* 2006). This allows real-time monitoring of vesicles in the sample chamber whilst undergoing either hydration or dehydration (Perrie *et al.*, 2007).

ESEM is possible due to a multiple aperture graduated vacuum system (as opposed to the single pressure limiting aperture used in SEM which introduced a high vacuum constraint on samples) shown in Figure 7.1, which maintains the electron gun at high vacuum (10^{-7} Torr), while the level of vacuum become progressively lower further down the column (Mathews *et al.*, 1999). The sample chamber can be kept at a constant pressure (usually in the range of 1.0–10.0Torr) thus allowing specimens to be imaged under water vapour or other gases, such as nitrogen or nitrous oxide (Dragnevski and Donald, 2008; Perrie *et al.*, 2007). The pressure limiting apertures allow the electron beam to travel between zones pumped at various rates, at the same time as minimising gas leakage to an acceptable level (Mathews *et al.*, 1999).

By means of a correct pump-down procedure and by controlling sample-temperature via a Peltier stage, dehydration can be suppressed therefore allowing specimens to be imaged in their natural state. Furthermore, by increasing the temperature of the sample or decreasing the pressure in the chamber, evaporation conditions within the specimen chamber can be achieved, which enables observation of the film-forming process (Dragnevski and Donald, 2008).

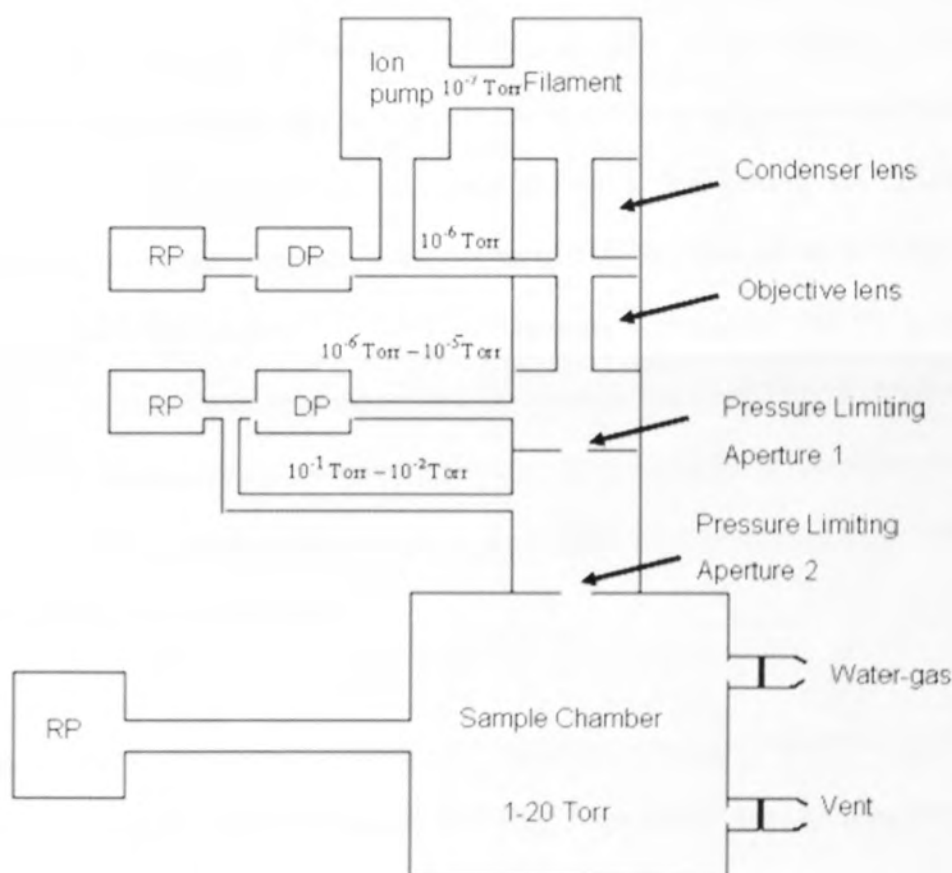


Figure 7.1 Schematic diagram of ESEM column, displaying the differential pumping zones. RP= rotary pump DP = diffusion pump (Adapted from Mathews et al., 1999)

ESEM has been utilised in the past to analyse hydrated samples: Cao and Li (2002) used the technique to monitor the effect of salt, alcohol and alkali on the interfacial

activity of a novel series of polymeric surfactant micelles based on carboxy methyl cellulose and alkyl poly(etheroxy) acrylate. ESEM micrographs revealed that the micelles could undergo disaggregation or be completely destroyed by the presence of the alkali. As a result, they observed the size of micelles to decrease significantly.

Within this study our aim was to investigate an alternative assay for determining stability of liposomes prepared to deliver solubilised low solubility drugs. The potential application of liposomes as therapeutic tools can still be limited by their innate physical and chemical instabilities, which can lead to an enhanced bilayer permeability and eventual loss of drug, vesicle aggregation/fusion, and precipitation (Mohammed *et al.*, 2007). To effectively deliver a formulation, the liposomal preparation must display an appropriate degree of stability. With the use of ESEM we have followed the real-time stability of liposomes during dehydration (gradual reductions in pressure or temperature) and investigated the effect bilayer composition has on this. By assessing the morphology and composition of liposomes under various conditions in the ESEM sample chamber, an alternative assay of vesicle formulation and stability was investigated.

Propofol was again selected as the model drug and incorporated within the liposomes since of all drugs studied for liposomal loading, formulations encapsulating propofol revealed the highest loading rates (Section 4.1.1).

7.1 The effect of drug-loading on liposome stability

Since the operating conditions of ESEM allow for the pressure and temperature of the chamber to be controlled and viewed in 'real-time' it would be interesting to follow

the changes in liposome morphology during temperature moderation whilst maintaining a near-constant operating pressure. With this aim, empty and drug-loaded DSPC liposomes (suspended in double-distilled water) were subjected to increasing temperatures and their morphology analysed using ESEM.

The samples were initially studied at a temperature of 15°C and 5.0Torr pressure. Micrographs obtained at this point revealed colonies of spherical structures for drug-loaded vesicles (Figure 7.2a) with a size distribution in range of 5-7µm in line with size analysis performed via laser diffraction (Section 5.1.1). Upon gradual increase of temperature to 20°C (at 5.0Torr pressure), the drug-loaded liposomes maintained their spherical morphology (image not shown). The propofol-entrapped vesicles appeared to remain intact at a further increase in temperature (to 25°C) (Figure 7.2b). At 15°C, drug-free formulation was observed as intact and spherical (Figure 7.3a). However, the increase in temperature to 20°C resulted in the empty liposomes losing their spherical shape as widespread flattening and spreading occurred, to yield lipid patches (Figure 7.3b).

The increase of temperature resulted in loss of the aqueous phase which coincided with coalescing of drug-free specimen while drug-loaded vesicles were shown to be resistant to temperatures as high as 25°C. This shows that the presence of drug appears to induce a difference in bilayer mechanics manifesting as a possible positive influence of propofol on bilayer stability.

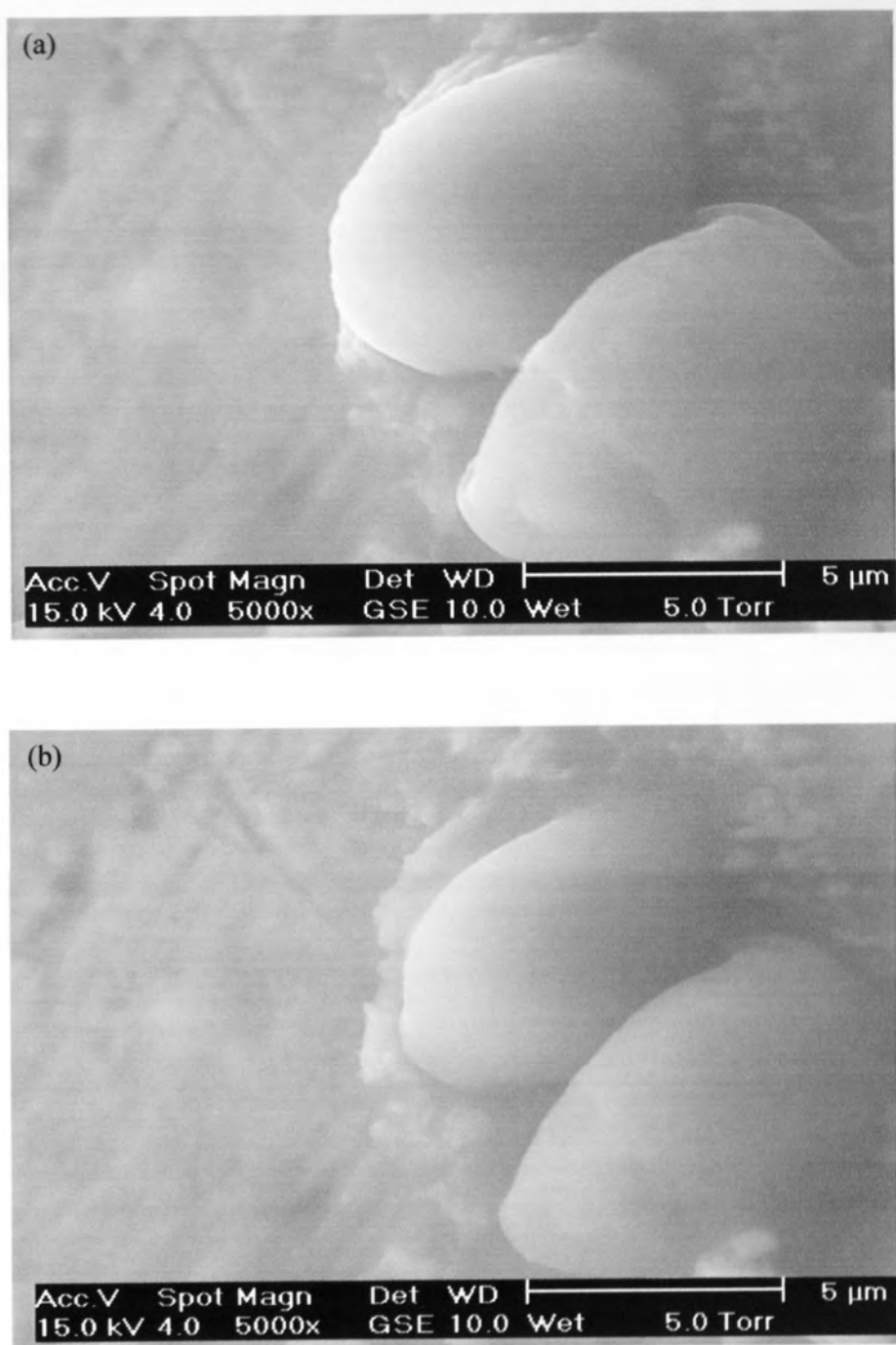


Figure 7.2 Environmental scanning electron micrographs of propofol-loaded liposomes (DSPC: cholesterol, 0.89: 0.11 molar ratio) suspended in double-distilled water. Vesicles were subjected to controlled increases in operating temperature in the ESEM sample chamber at constant operating pressure (5.0Torr). At a temperature of 15°C, liposomes appeared as spherical (a). Upon increase of the ESEM operating temperature to from 15°C to 25°C, did not influence propofol-incorporated MLV shape (b).

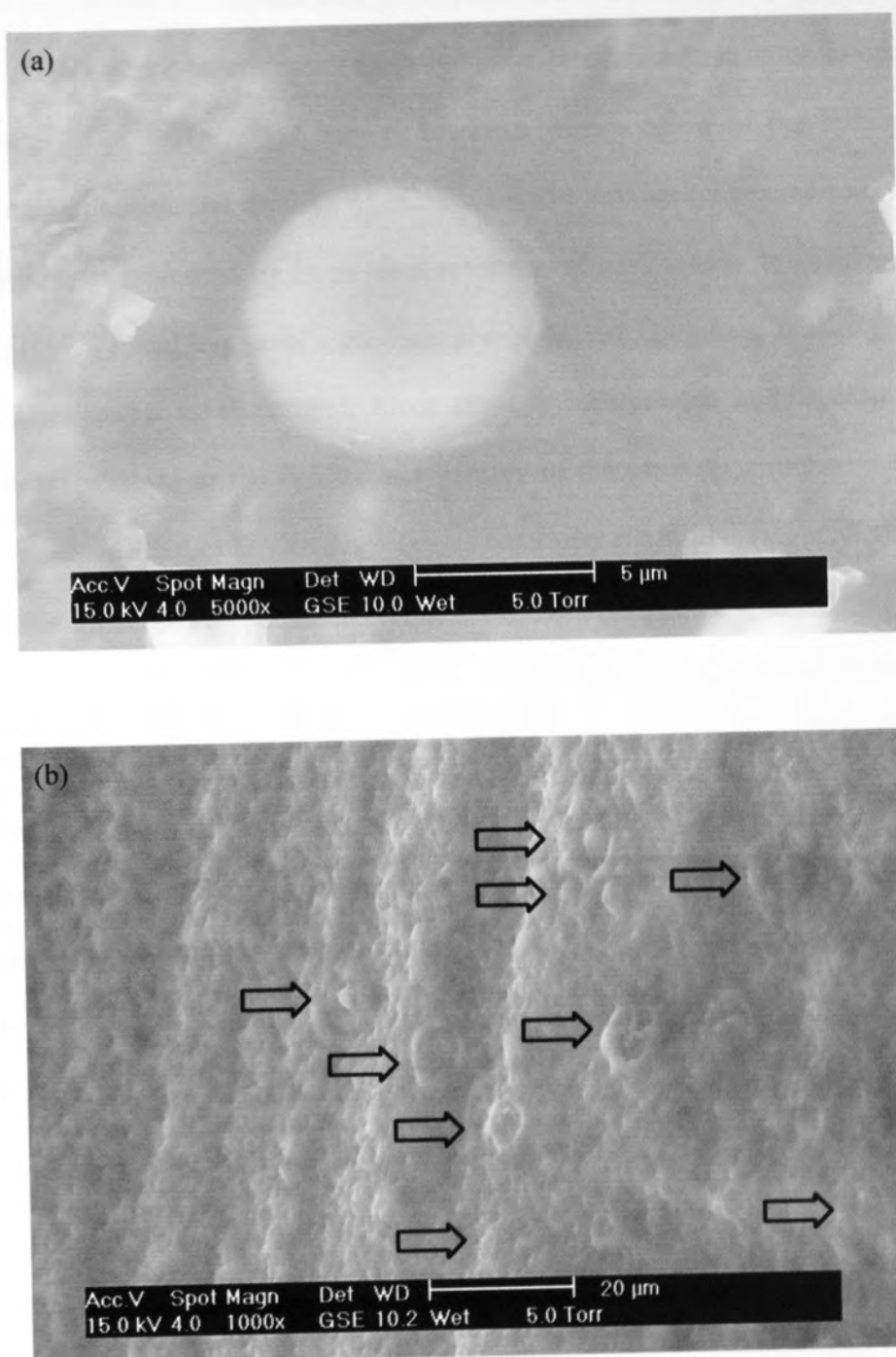


Figure 7.3 Environmental scanning-electron micrographs of drug-free liposomes (DSPC: cholesterol, 0.89: 0.11 molar ratio) suspended in double-distilled water. Vesicles were subjected to controlled increases in operating temperature in the ESEM sample chamber at constant operating pressure (5.0Torr). At a temperature of 15°C, drug-free liposomes appeared as spherical (a). Upon increase of the ESEM operating temperature to from 15°C to 20°C, vesicles collapsed and flattened to reveal a thin film (shown by arrows) (b).

The results appear to correlate with those published in a recent study by Mohammed *et al.* (2004) where under controlled dehydration (via gradual reduction in operating pressures from 4.0Torr to 1.9Torr), liposomal incorporation of the hydrophobic ibuprofen enhanced the stability of PC: cholesterol vesicles (compared to drug-free liposomes) as evidenced by an elevated resistance to coalescence. In addition, Shieh *et al.* (1996) found that taxol's presence also stabilised liposomes, probably via the same mechanism as cholesterol. Since taxol is incorporated in bilayer, it could possibly contribute to the rigidity and stability of the vesicular structure. However they concluded that excessive quantities of taxol may result in drug aggregation and precipitation of liposomes.

Improved stabilisation may not just be exclusively attributed to the presence of drug molecules. Vangala *et al.* (2006) demonstrated that the encapsulation of antigen by 1-monopalmitoyl glycerol multi-lamellar dehydration-rehydration vesicles could enhance stability of the vehicle as evidenced by increased resistance to coalescence whilst undergoing dehydration. They attributed this to amplification in bilayer rigidity as a result of the macromolecular antigen embedding itself in the spacing dividing adjacent lipid acyl chain moieties of the bilayers.

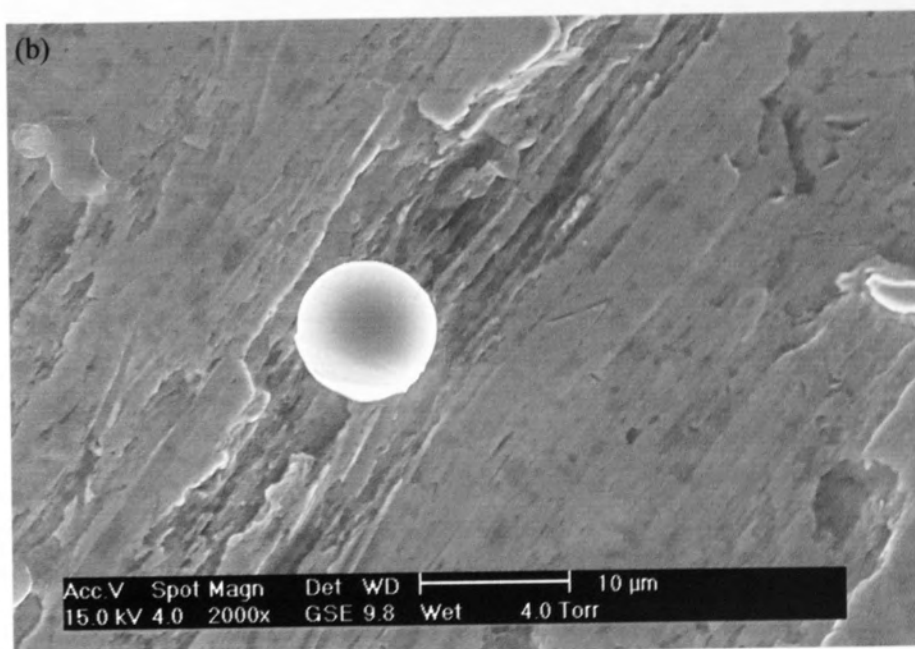
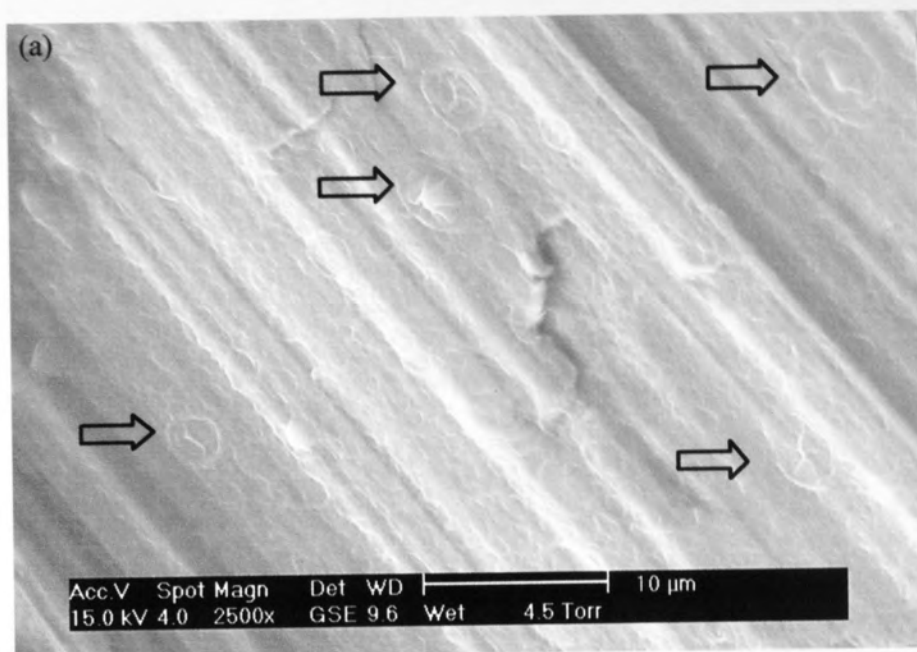
7.2 The effect of a near-fully charged bilayer on liposome morphology

Cationic liposomes showed enhanced drug encapsulation values compared to neutral preparations (Section 5.5.1). The DSTAP: cholesterol sample encapsulated significantly more (by ~10%) propofol when compared with DSPC: cholesterol formulation, a neutral preparation. These two systems were thus selected for assessing additional morphological characteristics and bilayer stability using the Philips XL-30

field emission gun ESEM. To illustrate the influence of a near-fully charged bilayer on the morphology of propofol-loaded vesicles formulated using DSTAP: cholesterol (0.89: 0.11 molar ratio), controlled dehydration was carried out via gradual reductions in pressure with comparisons made against its 18-carbon alkyl chain length zwitterionic analogous formulation, DSPC: cholesterol (0.89: 0.11 molar ratio) liposomes (both suspended in double-distilled water).

Investigations were initiated at a pressure of 4.5Torr and at a constant operating temperature of around 5°C. Initially samples containing the charged liposomes revealed fully-formed liposomes, but it was not possible to get photographs of these vesicles since upon concentration of the laser beam on the selected region to be examined in order to obtain a micrograph, the liposomes were observed to instantaneously collapse and flatten (Figure 7.4a). Therefore an ESEM image of the vesicles prior to collapse was not possible. Liposomes composed of DSPC did not show the same properties and could be visualised as clear spherical vesicle (Figure 7.4b). The size of the liposomes from the micrographs (7-8µm, Figure 7.4b) resembled the size measurements using laser diffraction ($8.52 \pm 1.12 \mu\text{m}$).

Reductions of the operating pressures from 4.5Torr to 2.4Torr, revealed the liposomes starting to flatten (Figure 7.4c); further reduction to 1.1Torr showed no further morphological alterations of the neutral formulation (Figure 7.4d). The results suggest the DSTAP: cholesterol: propofol formulation was more unstable under the conditions induced in the ESEM chamber compared to their neutral counterpart, DSPC: cholesterol: propofol. This may be due to a less rigid and less compact bilayer which would result in repulsion between cationic head-group of the charged lipid.



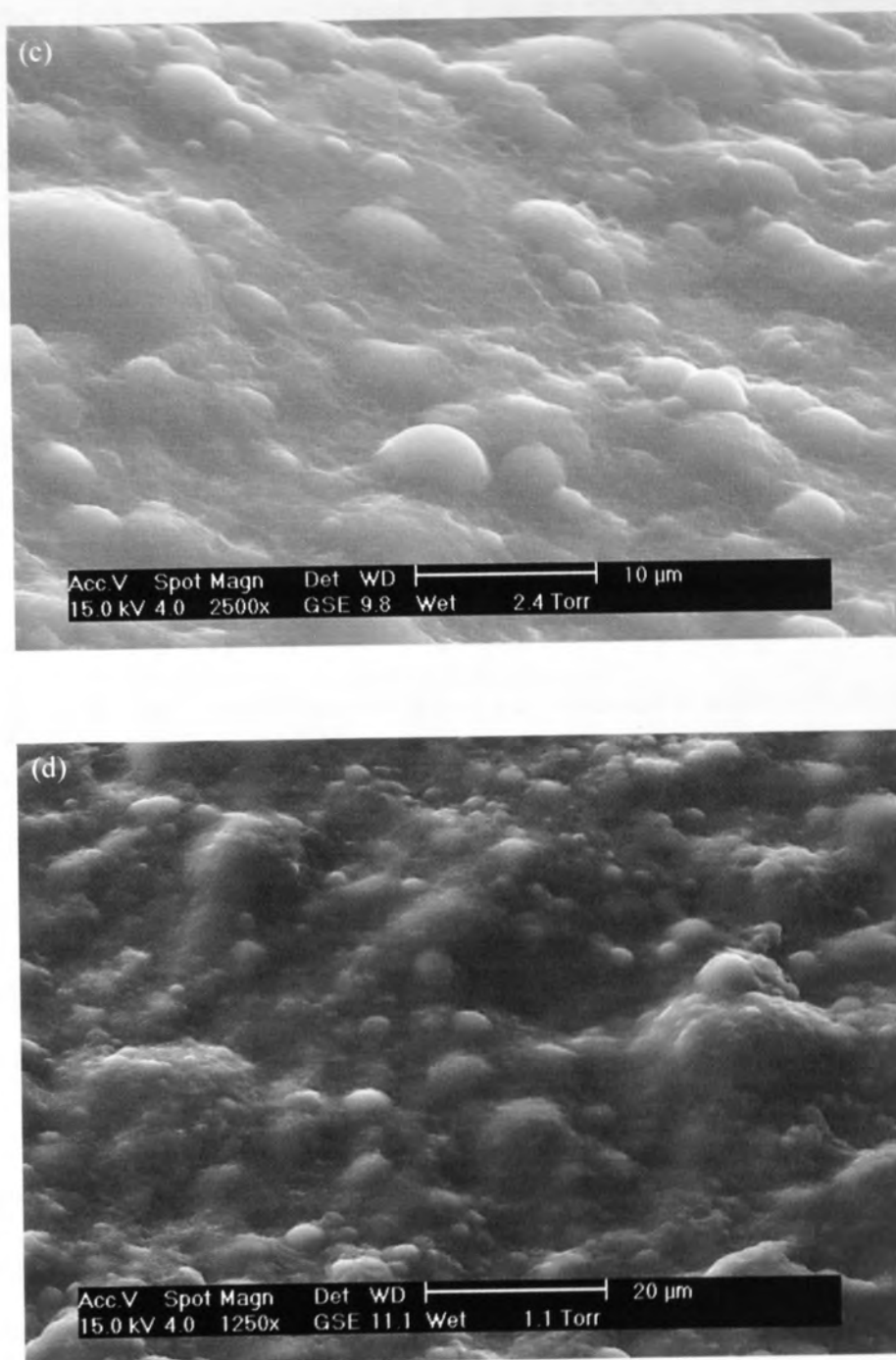


Figure 7.4 Environmental scanning-electron micrographs of propofol-encapsulated charged and neutral vesicles suspended in double-distilled water. Vesicles were subjected to controlled dehydration in ESEM sample chamber at constant temperature. At an operating pressure of 4.5Torr, charged liposomes (DSTAP: cholesterol, 0.89: 0.11 molar ratio) were observed to instantaneously collapse and flatten (indicated by arrows) upon concentration of the laser beam on the selected region to be examined (a). At pressure of 4.5Torr, neutral liposomes (DSPC: cholesterol, 0.89: 0.11 molar ratio) were seen as spherical structures (b). At reduced pressures of 2.4Torr (c) and 1.1Torr (d) the liposomes are seen to flatten.

7.3 Effect of cholesterol concentration on liposome stability

Preliminary optimisation studies (Section 4.1.1) showed that increasing cholesterol content reduced drug-encapsulation capabilities of liposomes and progressively decreased the cumulative release of propofol from the vesicles. This data suggested that there is a competitive interaction between drug loading in the liposomal bilayer and cholesterol bilayer content. ESEM was used to follow the dynamic stability of vesicles during dehydration and the effect bilayer composition has on this.

The effect of cholesterol concentration on liposome morphology and stability was examined. A DSPC (16 μ mol) formulation with cholesterol concentration of 20 and 33 total molar % (with propofol encapsulation) was subjected to controlled dehydration in the ESEM sample chamber and compared with results for sample containing 11 total molar % cholesterol displayed in Section 7.2, whilst maintaining the operating temperature at about 5°C throughout. Earlier (Section 7.2), DSPC formulation prepared with inclusion of 11 total molar % cholesterol revealed intact and fully-formed vesicles on reduction of operating pressures from 4.5 to 1.1Torr. Initially, at an operating pressure of 4.0Torr, micrograph for DSPC preparation containing 20 total molar % cholesterol show vesicles covered in a blanket of moisture as dehydration was in process although some spherical structures of liposomes were beginning to appear (Figure 7.5a). Upon reduction of the pressure to 2.4Torr, vesicles appeared as clear spherical vesicles that resisted any significant morphological deviation (Figure 7.5b). With a further reduction of the ESEM operating pressure from 2.4Torr to 1.1Torr revealed spherical structures as well as some flattening spherical structures (Figure 7.5c).

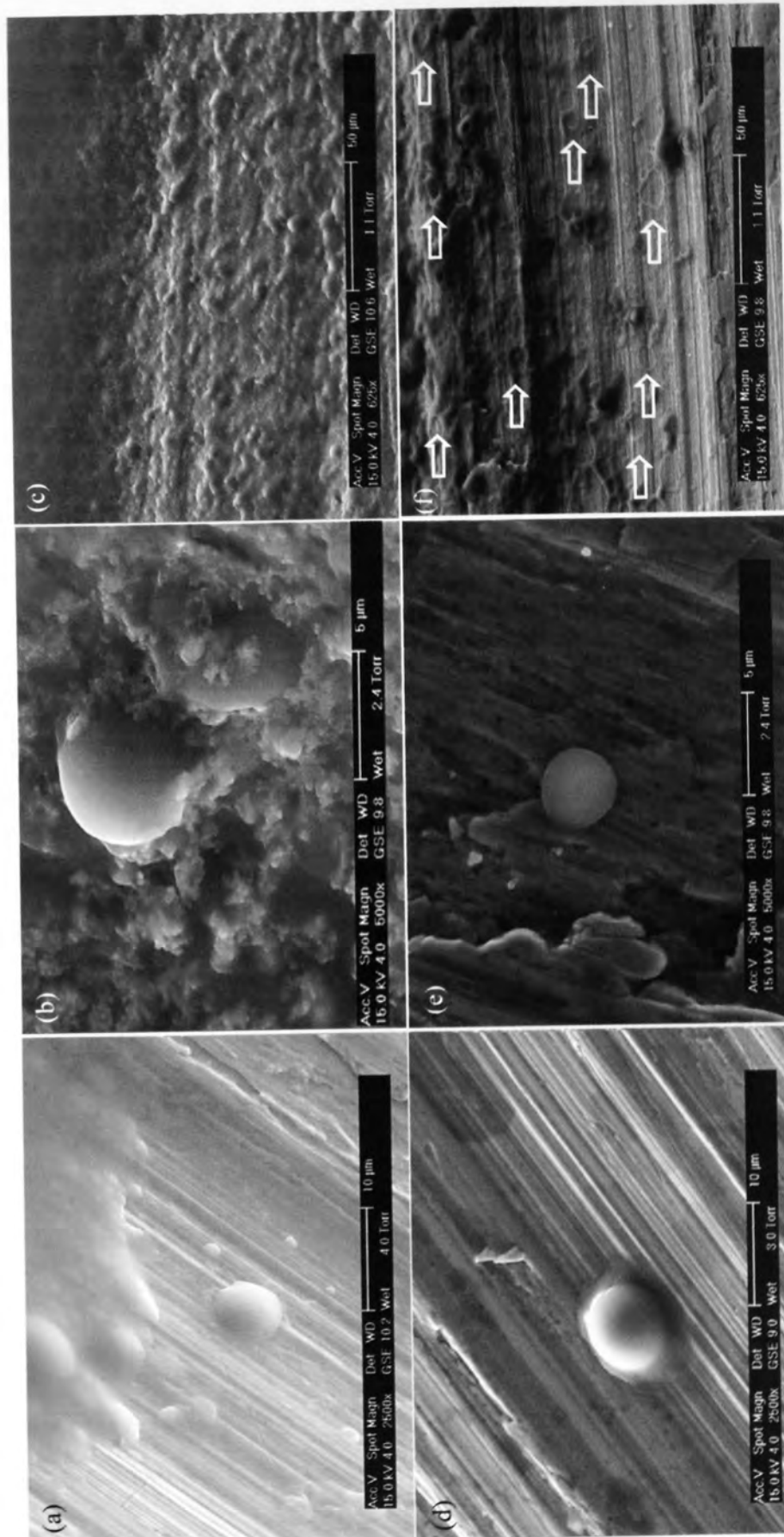


Figure 7.5 Environmental scanning electron micrographs of dry propofol-loaded DSPC lipid films containing 20 total molar % (a-c) and 33 total molar % cholesterol (d-f) samples suspended in double-distilled water. Both formulations were subjected to controlled dehydration in the sample chamber and the 20 total molar % cholesterol-containing liposomes were shown to be resistant to pressure as low as 1.4Torr, as opposed to 33 total molar% cholesterol-containing preparation which was observed to coalesce (represented by arrows) at pressure reduction to 1.4Torr (f).

At an initial operating pressure of 3.0Torr (due to existing humidity within ESEM sample chamber from previous experiments) for formulation containing 33 total molar % cholesterol, liposomes were observed as clear, well-defined spherical structures (Figure 7.5d), that were also seen in images obtained at a lower operating pressure of 2.4Torr (Figure 7.5e). However, with liposome containing 33% cholesterol there is very few such structures present with the majority of the liposomes appearing to have collapsed at operating pressure of 1.1Torr (Figure 7.5f).

The results (Figure 7.5) suggest DSPC formulation prepared from the inclusion of 33 total molar % cholesterol was not as stable as the formulations prepared from 11 and 20 total molar % cholesterol under the ESEM environments studied. The ESEM analysis do not appear to relate to previous studies (Deol and Khuller, 1997; Kirby and Gregoriadis, 1984) that showed increasing cholesterol content of liposomes of up to 50 total molar % yield stable drug-loaded formulations. Based on evidence here, ESEM may not appear to be a very reliable tool as a standalone in certain situations; however it could be used in conjunction with a range of other tools.

7.4 Effect of alkyl chain length on liposome stability

Previous results (summarised in Table 7.1) revealed a general increase in propofol incorporation with increasing lipid alkyl chain length in the order of $C_{24}PC > DSPC > DMPC \geq DPPC = PC$ ($P < 0.05$) which could be attributed to increased hydrophobic regions within the bilayer for the hydrophobic propofol to interact with; a general increase in the cumulative release rate with descending lipid chain length was observed for subjects stabilised with 11 total molar % cholesterol in the order of $PC > DMPC = DPPC > DSPC$ following 72hour study at 37°C ($P < 0.05$). As stated earlier

in Section 5.1.3, bilayer drug release is recognised to be influenced by the phase transition temperature (T_c) of the lipid excipients within the vesicles, which is significantly influenced by the alkyl hydrocarbon chain length (Mohammed *et al.*, 2004). Preliminary stability studies performed in Section 5.1.4 suggested stable formulations that shared common stability profiles up to 28day of investigation.

Parameter	Lipid alkyl chain length (no. of carbons)		
	14	16	18
Encapsulation (% mol/mol)	22.4 ± 1.6	19.8 ± 1.1	24.8 ± 1.5
Cumulative release after 72hour (%)	41.1 ± 1.0	39.2 ± 1.2	33.5 ± 2.0
Size (μm)	7.8 ± 0.9	8.7 ± 1.0	8.5 ± 1.1

Table 7.1 Comparison of characteristics of PC formulations prepared from phospholipids of varying cholesterol content (and 1.00mg propofol). Results are expressed as the means of at least four experiments \pm S.D. (Data for encapsulation and size were previously presented in Section 5.1.1 and release displayed in Section 5.1.3, are presented here for ease of comparison)

To further investigate the role of the alkyl chain length on the stability offered during dehydration of the MLV, propofol-encapsulated liposomes prepared from DMPC or DPPC (with addition of 11 total molar % cholesterol) suspended in double-distilled water were investigated, and compared with results for DSPC sample containing 11 total molar % cholesterol presented in Section 7.2.

To begin with, the liposome samples were examined at an operating pressure of 4.0 to 4.5Torr and at a constant temperature of around 5°C. The micrographs obtained under these conditions (e.g. DMPC liposomes; Figure 7.6a) revealed clear, spherical vesicles that were similar in nature with no apparent influence by the difference in hydrocarbon chain length being able to be differentiated using this method. The size of the liposomes from the images generally resembled the mean volume diameter collected via laser diffraction.

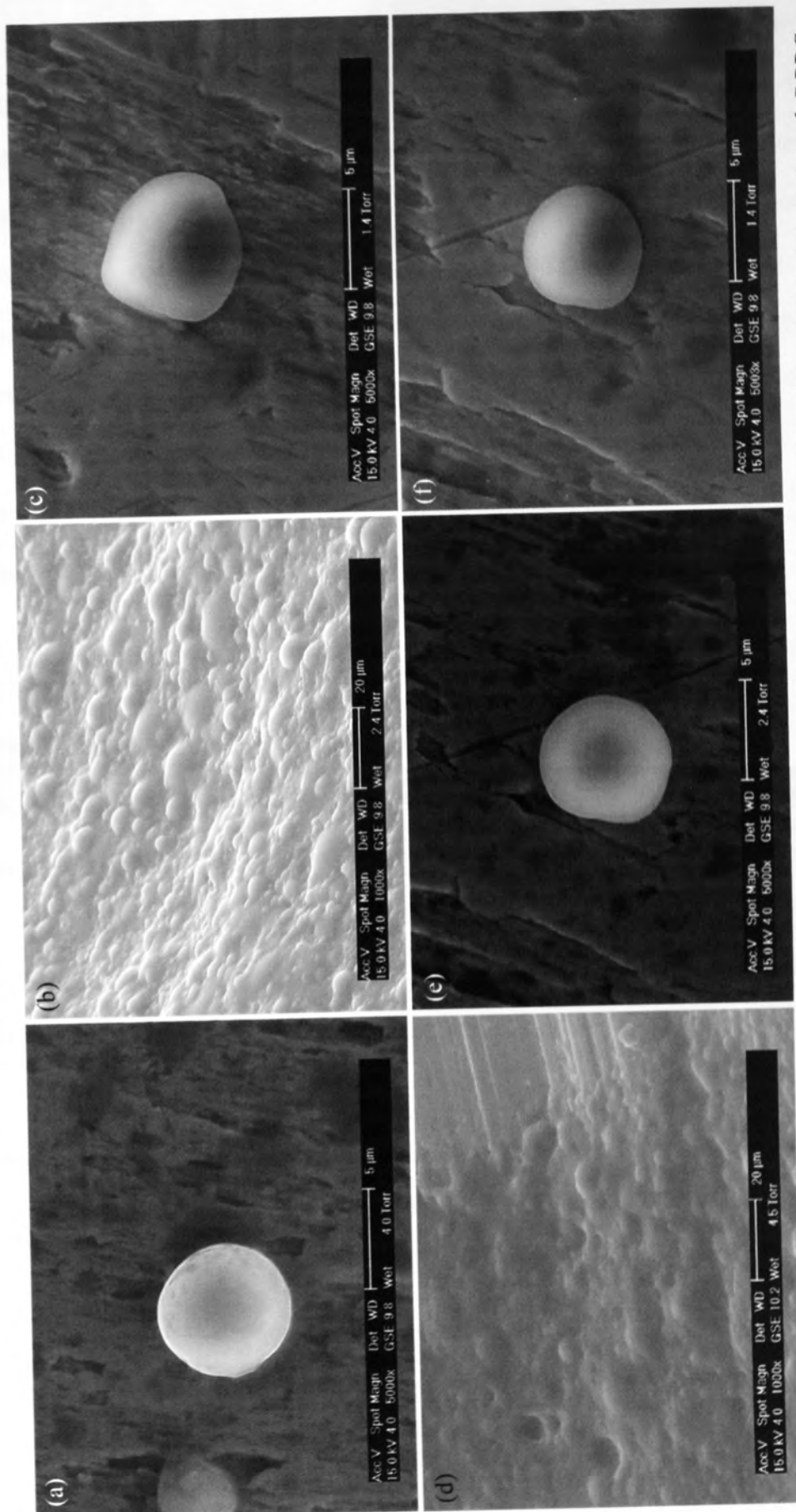
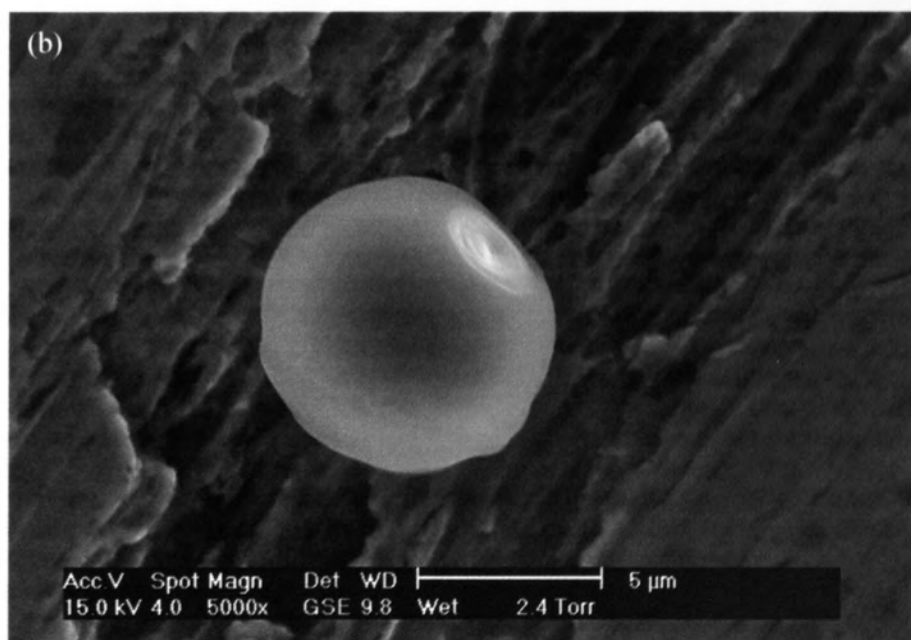
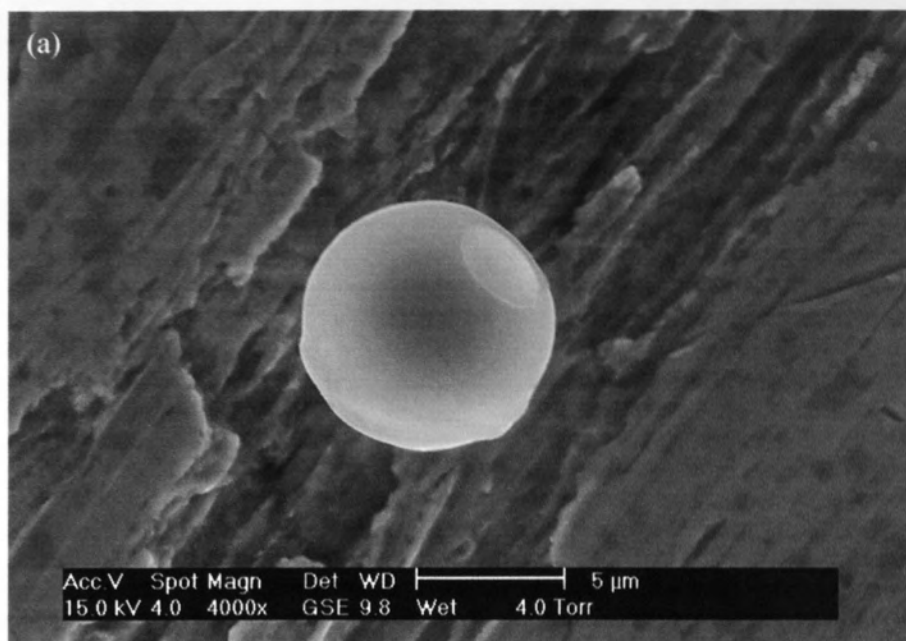


Figure 7.6 Environmental scanning electron micrographs of dry propofol-loaded lipid films of DMPC: cholesterol (a-c) and DPPC: cholesterol (d-f) specimens suspended in double-distilled water. Both formulations were subjected to controlled dehydration in the sample chamber and the liposomes were revealed to be resistant to pressure as low as 1.4Torr.

Reductions of operating pressures to 2.4 and 1.4Torr displayed no signs of vesicular morphological changes (example for DMPC at 2.4 and 1.4Torr revealed in Figures 7.6b and c, respectively). The liposomes in all of the three samples were generally intact, spherical and resisted any deviation during the pressure adjustments. Similar to DMPC and DSPC (Section 7.2) formulations, DPPC: cholesterol (Figures 7.6d-f) specimen suspended in double-distilled water was also revealed to be resistant to pressure as low as 1.4Torr. The results generated indicate the formation of stable propofol-loaded liposomes by DSPC, DPPC and DMPC phospholipids in the presence of 11 total molar % cholesterol concentration. Alkyl chain length does not appear to affect the stability of liposomes under the conditions studied here.

7.5 The role of geometry on liposomal morphology

The influence of geometry on liposomal morphology and stability was studied under controlled dehydration via operating pressure reductions. POPC, which is structurally asymmetrical, was investigated with comparisons generated with previous results (Section 7.4) obtained for their symmetrical counterparts, DMPC, DPPC and DSPC. All formulations were prepared with 11 total molar % cholesterol concentration. Initial examination was performed at a pressure of 4.0Torr at which point micrographs generated revealed clear spherical vesicles (Figure 7.7a). Operating pressure drop from 4.0 to 2.4Torr, and then to 1.4Torr did not affect the morphology of the liposomes since they retained their spherical structure (Figure 7.7b and c, respectively). Temperature in the ESEM chamber was maintained at around 5°C throughout. Asymmetric vesicle sizes obtained from ESEM images were in agreement with the particle size analysis (Section 5.2.2).



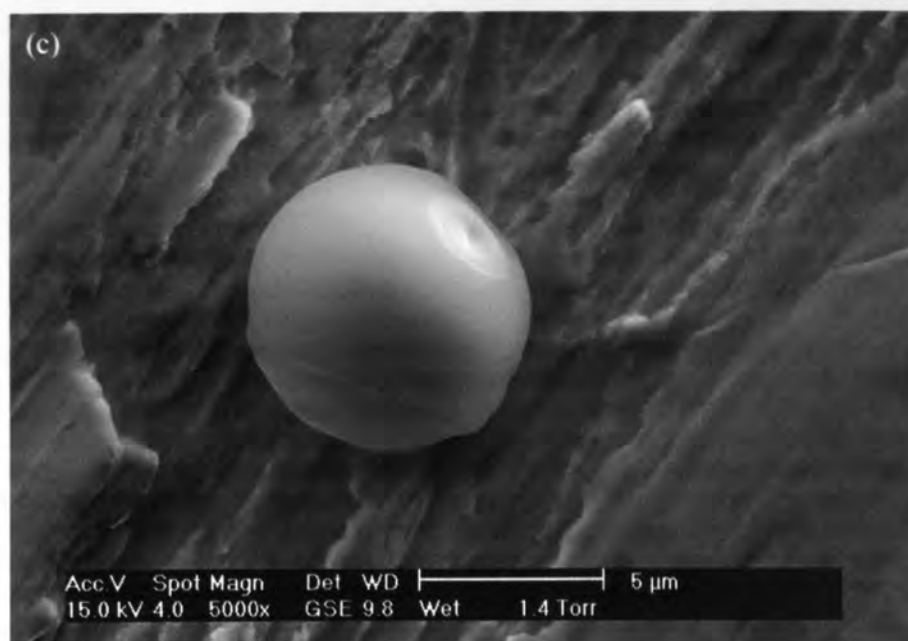


Figure 7.7 Environmental scanning electron micrographs of propofol-loaded liposomes formed using POPC and 11 total molar % cholesterol suspended in double-distilled water. Vesicles were subjected to controlled dehydration in the ESEM sample chamber. At an operating pressure of 4.0Torr, vesicles appeared as spherical structures (a). Gradual decrease of the operating pressure to 2.4 and 1.4Torr showed intact, stable liposome structures that evidently resisted any significant morphological changes (b and c, respectively).

7. 6 Conclusions

Environmental scanning electron microscopy enabled us to view fully hydrated, chemically unaltered samples and conclude various interesting features of the relationship between numerous aspects of liposomes and their physical stability and integrity in real-time.

ESEM studies have allowed us to visualise MLV in their hydrated condition without any further processing prior to imaging. The ability to control pressures and temperatures allowed us to vary these aspects and monitor the changes in the liposome morphology over time. It is clear that liposomes in the ESEM chamber are susceptible to collapse under reduced pressures and that the liposome composition does play a role in dictating the resistance to such collapse. Indeed the more gaseous-

type bilayer of the highly cationic DSTAP liposomes were very unstable under ESEM examination and were difficult to image effectively. However the neutral liposome formulations such as DSPC and POPC showed good stability and interesting images were produced using this technique. However the applicability of using this system as a new method to investigate liposome stability appears less valid than first hoped since the results are often open to varied interpretation and do not provide a robust set of data to support conclusions in certain cases. Indeed some of the findings here are contradictory to previously, well-established knowledge regarding liposome stability and show little correlation (such as the formulation containing 33% cholesterol collapsing; Section 7.3). However the stress conditions the liposomes are subjected to during dehydration/reduction of pressure in the ESEM chamber may better be suited to investigations into freeze-drying of liposomes and therefore further work could be continued in this respect.

Furthermore, using a technique like laser diffraction, variation in vesicle size between different formulations is obtainable since the tool determines an average value of vesicles in specimen. However these changes are small and unlikely to be noted in most microscopy methods, further highlighting the limitations of a method such as the ESEM.

Chapter Eight:

Using small angle neutron scattering to assess bilayer mechanics

8.0 Introduction

Work thus far has substantiated key parameters controlling entrapment of poorly water-soluble drugs within liposomal bilayers in order to exploit liposomes as vehicles for the delivery of drugs of this nature. It has already been revealed that encapsulation and release of sparingly water-soluble drugs into PC liposomes is affected by the alkyl chain length of the phospholipids comprising the liposomes (e.g. DSPC). The research performed to date has also shown that encapsulation is also dependent on the presence and concentration of cholesterol.

Earlier studies showed that drug incorporation increases with increase in the alkyl chain length of phospholipid comprising the liposomes. Results in Section 5.1.1 revealed an increase in midazolam and propofol bilayer-loading with an increase in lipid chain length thus supporting previous work (Mohammed *et al.*, 2004). As discussed in Section 5.1.1, this trend could be attributed to the possible increased bilayer lipophilic area within systems formed by these longer lipid bilayers for the hydrophobic drug to accommodate within. This theory is backed by Dan (2007) who cites that bilayer thickness increases with increasing alkyl chain length of phospholipids, a premise also suggested by Mohammed *et al.* (2004). Determination of the changes in bilayer thickness upon increase in the length of main lipid component with and without the presence of drug will therefore help confirm this hypothesis.

There are also some reports that have observed an enlargement in the mean vesicular sizes upon encapsulation of hydrophobic drugs (e.g. Mohammed *et al.*, 2004), or cholesterol (e.g. Gallova *et al.*, 2004) within the bilayer although studies in the

previous sections here largely have not correlated with this. It would be therefore be instructive to further investigate this theory.

Therefore to study this the influence of phospholipid alkyl chain length, presence of drug and inclusion of cholesterol on liposome bilayer thickness, lamellarity and interlamellar spacing using the LOQ spectrometer at the ISIS facility (Rutherford Appleton Laboratory, Chilton, UK) (Figure 8.1) was attempted. Determination of the bilayer thickness is essential for the characterisation of parameters affecting bilayer interactions in membranes (Balgavy *et al.*, 2001).

8.1 Small angle neutron scattering- a brief literature review

Small-angle neutron scattering (SANS) is a technique accessible at neutron-scattering facilities requiring a neutron source, i.e. a nuclear reactor or an accelerator-based spallation source (such as the one at the ISIS Facility). For over 60 years, SANS, preceded by SAXS (small-angle X-ray scattering), has been used to investigate materials such as the microstructure of alloys, ceramics, polymers and colloids (Radlinski, 1999).

Light and, to a lesser degree, X-ray scattering are extensively utilised in the pharmaceutical industry to study the structure and mechanisms of various drug delivery systems that are amenable to study by SANS such as colloidal and polymeric vehicles. Small angle neutron scattering (SANS) has the potential to yield complimentary and, frequently, more comprehensive data regarding drug delivery systems, however is far less regularly exploited (Lawrence and Barlow, 2006).

Neutrons have wavelengths that are comparable to X-rays (i.e. in the range of 1-10 Å) and hence scattering experiments can cover the atomic to macromolecular scale as well as the smaller end of the colloidal size range. The interaction of neutrons with matter is relatively weak and consequently they do not perturb the test system allowing them to penetrate sufficiently to probe the bulk characteristics of samples (Lawrence, 2002).



Figure 8.1 A view of the LOQ sample position. The neutron beam originates from the long silver pipe in the core, having passed through a chamber comprising the collimator and incident beam monitor (a). A photograph of the Experimental Hall, ISIS, Rutherford Appleton Laboratory, Chilton, Oxford (b).

The general purpose of a SANS experiment is to establish the differential cross-section in order to yield data on the shape, size and interactions of the scattering bodies (congregation of scattering centres) in the test sample (Magazu *et al.*, 2008). The source neutrons interact with the nuclei of the species in the sample (Figure 8.2) and this interaction is dependent on the isotope used for study; hydrogen and deuterium are typically utilised as they have very different scattering length and this difference is important in contrast variation experiments where a portion of the molecule in solution is usually deuterated (Segota and Tezak, 2006) with the scattering length density of the molecule being determined by its atomic composition

(Gilbert *et al.*, 1999). The scattered neutrons are collected for each scattering angle. The scattering intensity is obtained as a result of the interference of spherical waves originating from the nuclei that pertain to the same or different molecular species. Corrections for inelastic influences, multiple scattering, absorption, effects of sample container and background are normally taken into account (Magazu *et al.*, 2008).

The standard way of analysing data obtained from experiments using SANS (see, e.g. Magazu *et al.*, 2008) involves modelling of the sample structure by assuming several theoretical models, and of calculating the corresponding scattering curves. Thereafter, the calculated curves are contrasted with the experimental data, and the validity of the model can be confirmed via the analysis of the consistency of the fitted curves with the collected data (Almasy *et al.*, 2005).

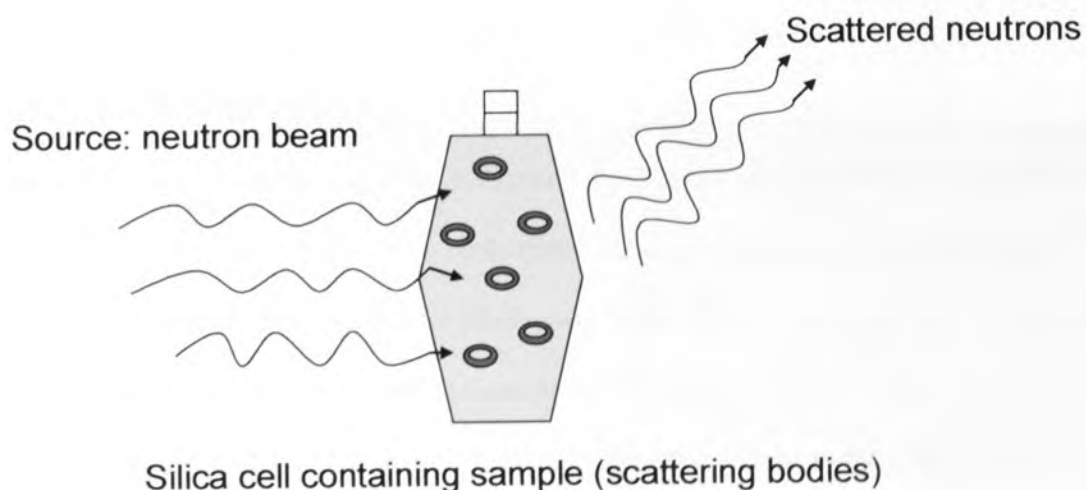


Figure 8.2 A schematic illustration of a small angle neutron scattering experiment showing the scattering behaviour of the source neutrons following interaction with nuclei of test sample (adapted from Lawrence, 2002).

Practically, sample preparation for SANS is much simpler than when used for analysis via light scattering as dust becomes less of an obstacle. Furthermore, SANS studies can be performed in solvents that equate more to physiological conditions with

much reduced volumes of the test sample required for analysis (Lawrence, 2002). At the very least, in fully-deuterated solvent, data on the size and shape of, for example, a colloidal particle like the neutron scattering data of liposomes can be obtained. For example, Nowrath *et al.* (2004) found that the magnetic shell of liposomes were typically 100–400nm in size, as required for applications *in vivo*.

Even though the use of SANS in drug delivery research is still limited (Lawrence and Barlow, 2006), there are sufficient published data (e.g. Harvey *et al.*, 2007; Pencer *et al.*, 2005; Uhrikova *et al.*, 2000) to demonstrate the potential of the procedure in drug delivery exploration, when used as a standalone method or in amalgamation with other more established characterisation methods. For example, Balgavy *et al.* (2001) concluded that a joint analysis of SANS and SAXS on unilamellar liposomes can be used to collect the value for bilayer thickness and the distance of the lipid phosphate head-group from the bilayer hydrocarbon moiety. In another instance, model fitting of the SANS data following studies performed by Harvey *et al.* (2005) on vesicles prepared from the non-ionic surfactant 1,2-di-*O*-octadecyl-*rac*-glyceryl-3-(ω -methoxydodecaethylene glycol), in deuterated water (D₂O) solutions with various ionic strengths of LiCl, NaCl and NaI, revealed alterations in bilayer parameters, i.e. thickness and repeat spacings, in response to the presence of ions in the bulk media.

Using SANS to study the influence of cholesterol has also been carried out in the past. Pencer *et al.* (2005) performed SANS measurements on pure DMPC liposomes and those comprising of cholesterol, ergosterol or lanosterol (sterols). Data generated revealed at temperatures ranging from 10 to 45°C, the presence of the various sterols resulted in the increase in bilayer thickness compared to formulation without any of

the sterols. Moreover, Gallova *et al.* (2004) cite the increase in bilayer thickness in DMPC specimens following addition of cholesterol.

8.2 The experimental set-up

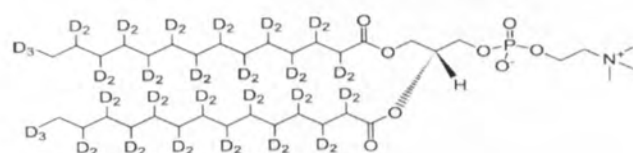
Liposome samples were prepared for SANS via the established hydration method described in Section 2.2.1 and subsequently characterised by light scattering and zeta potential prior to study by SANS (Table 8.1). The SANS experiment was performed by using the LOQ spectrometer at the ISIS facility on hydrogenous (*h*-) liposome/D₂O mixtures and fully or partially deuterated (*d*-) liposome/H₂O mixtures. To determine whether the structure of the bilayer and the overall size of vesicles prepared from lipids of varying alkyl chain length (C₁₄, C₁₈ and C₂₄) was distorted by the addition of cholesterol or drug (ibuprofen) or both, SANS experiments were carried out as follows (*d*-drug was unavailable; three contrasts were used for DMPC and DSPC while only one was used for C₂₄PC due to cost):

- 1) *h*-lipid (DMPC, DSPC or C₂₄PC) in D₂O;
- 2) Chain *d*-lipid (DMPC[PC D₅₄] or DSPC[PC D₇₀] in H₂O;
- 3) *d*-lipid (DMPC[PC D₆₇] or DSPC[PC D₈₃]) in H₂O.

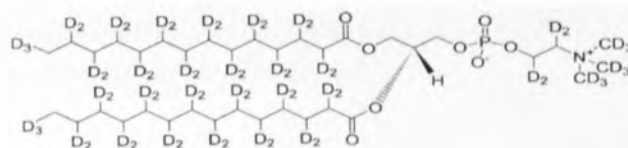
Test 1 was performed to obtain information regarding the size and shape of the vesicles whilst tests 2 and 3, were carried out to complementarily provide information regarding bilayer thickness, lamellarity and interlamellar spacing. For comparative intentions, a parallel experiment was also performed on liposomes formulated from

respective pure *h*-lipid only (without drug or cholesterol), under replica experimental conditions. Formulations were prepared in the composition lipid: cholesterol, 0.89:0.11 molar ratio with the addition of 1.0mg drug (where applicable). The fully- and partially- deuterated structures are shown in Figure 8.3 and formulation compositions in Table 8.1.

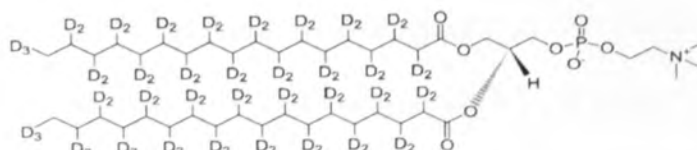
Preparations were placed in meticulously cleaned disc-shaped fused silica cells of path length 2mm. Using a 12mm diameter neutron beam, measurements were performed at an electronically controlled sample temperature of 298K, with typically 40-min exposure. Backgrounds from pure D₂O, were subtracted as appropriate.



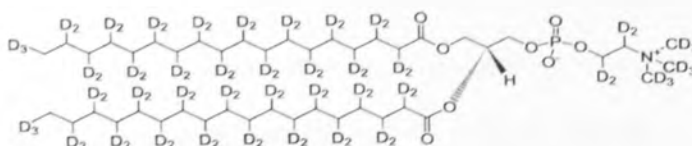
DMPC(PC D₅₄)



DMPC (PC D₆₇)



DSPC (PC D₇₀)



DSPC (PC D₈₃)

Figure 8.3 Chemical structures of the fully- and partially-deuterated phospholipids (DMPC and DSPC) used for SANS studies.

Sample number	Formulation	Solvent	Size (μm)	Zeta potential (mV)
1	DMPC	D ₂ O	10.57	-2.4 \pm 4.4
2	DMPC: ibuprofen	D ₂ O	4.03	-3.3 \pm 5.3
3	DMPC: cholesterol	D ₂ O	6.02	5.6 \pm 8.2
4	DMPC: cholesterol: ibuprofen	D ₂ O	7.56	2.1 \pm 4.2
5	DSPC	D ₂ O	5.05	-2.9 \pm 2.0
6	DSPC: ibuprofen	D ₂ O	4.85	-4.7 \pm 7.2
7	DSPC: cholesterol	D ₂ O	6.14	2.2 \pm 4.6
8	DSPC: cholesterol: ibuprofen	D ₂ O	6.28	2.8 \pm 5.3
9	C ₂₄ PC	D ₂ O	5.65	-6.4 \pm 6.6
10	C ₂₄ PC: ibuprofen	D ₂ O	7.52	0.2 \pm 5.4
11	C ₂₄ PC: cholesterol	D ₂ O	5.20	4.4 \pm 4.3
12	C ₂₄ PC: cholesterol: ibuprofen	D ₂ O	9.93	5.2 \pm 7.4
13	DMPC (PC D ₅₄)	H ₂ O	6.34	-6.7 \pm 5.0
14	DMPC(PC D ₅₄): ibuprofen	H ₂ O	3.07	5.3 \pm 7.6
15	DMPC(PC D ₅₄): cholesterol	H ₂ O	5.52	7.2 \pm 7.9
16	DMPC(PC D ₅₄): cholesterol: ibuprofen	H ₂ O	5.81	-3.7 \pm 7.0
17	DSPC(PC D ₇₀)	H ₂ O	6.74	0.9 \pm 8.3
18	DSPC(PC D ₇₀): ibuprofen	H ₂ O	7.21	-2.9 \pm 4.6
19	DSPC(PC D ₇₀): cholesterol	H ₂ O	6.57	5.1 \pm 3.0
20	DSPC(PC D ₇₀): cholesterol: ibuprofen	H ₂ O	8.25	-1.3 \pm 4.6
21	DMPC(PC D ₆₇)	H ₂ O	6.03	-3.3 \pm 9.1
22	DMPC(PC D ₆₇): ibuprofen	H ₂ O	6.64	-6.5 \pm 7.8
23	DMPC(PC D ₆₇): cholesterol	H ₂ O	5.46	-5.9 \pm 6.4
24	DMPC(PC D ₆₇): cholesterol: ibuprofen	H ₂ O	4.35	1.1 \pm 5.8
25	DSPC(PC D ₈₃)	H ₂ O	7.83	4.5 \pm 5.5
26	DSPC(PC D ₈₃): ibuprofen	H ₂ O	6.41	-4.1 \pm 7.0
27	DSPC(PC D ₈₃): cholesterol	H ₂ O	6.70	6.5 \pm 5.7
28	DSPC(PC D ₈₃): cholesterol: ibuprofen	H ₂ O	7.63	0.9 \pm 4.2

Table 8.1 Formulation (lipid: cholesterol, where applicable, is 0.89: 0.11 molar ratio) description and vesicle size determination for samples analysed via SANS, $n=1$. Zeta potential values are represented as the average of 10 readings.

8.3 Results and discussion

The aims of the study were to investigate the response to variation in phospholipid alkyl chain length, presence of drug and inclusion of cholesterol on liposome bilayer thickness, lamellarity and interlamellar spacing using the LOQ spectrometer at the ISIS facility. However, analysis could not be completed due to possible liposomal stability-related issues. Many samples did not remain homogenous as noticeable creaming and flocculation of the MLV began to occur even at initial handling of the silica cells. This flocculation did not occur with samples left to stand in normal sample container over a similar time-frame. For example, separation took place in samples 4, 9 and 10, whilst samples 2, 3 and 8 were stable at least during the first 10

min (Figure 8.4). There was no consistency in the display in the separation behaviour since samples differing in parameters (i.e. presence of drug or cholesterol) revealed similar levels of separation. Samples could be resuspended with vigorous shaking however this could not be maintained over the time period of the sample run. A similar issue has been recently published by Harvey *et al.* (2007), although they did not state a possible explanation for the process. The SANS procedure is reported to be sensitive to the distribution of the species in a liquid suspension of small molecules (Almasy *et al.*, 2005). A heterogeneous arrangement of particulates should be avoided as it can lead to a variable distribution of the scattering length density, which results in a raise in small-angle scattering (Almasy *et al.*, 2005).

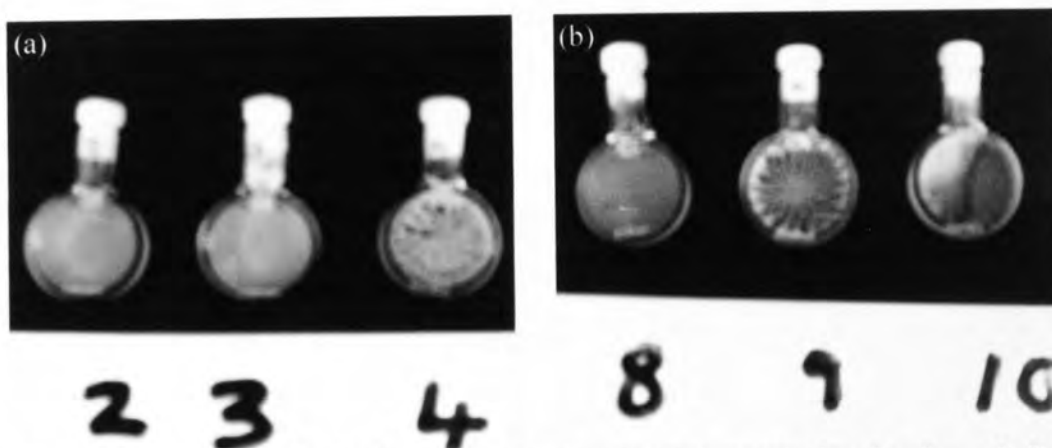


Figure 8.4 Images displaying preparations placed in meticulously cleaned disc-shaped fused silica cells of path length 2 mm: (a) DMPC (sample 2), DMPC:cholesterol (sample 3), DMPC:cholesterol:ibuprofen (sample 4); (b) DSPC:cholesterol:ibuprofen (sample 8), C₂₄PC (sample 9), C₂₄PC:ibuprofen (sample 10).

8.4 Conclusions

Although attempts here ultimately proved unsuccessful, the SANS technique could potentially provide a viable method to study bilayer mechanics. The problems encountered here could be overcome by examining small unilamellar vesicles rather than MLV as performed by Harvey *et al.* (2005).

Despite the unsuccessful attempt to obtain experimental data, literature reviewed here suggest that using SANS to assess bilayer mechanics could greatly aid in the rational development of liposome-based formulations as vehicles for poorly water-soluble drugs. By using appropriate fit models and analysis techniques, SANS is capable of yielding data on the shape and size of the scattering bodies in the liposome mixtures.

Chapter Nine:

General discussion and conclusions

9.0 General discussion and conclusions

Liposomes, which self-assemble into vesicles in an aqueous environment (Talsma *et al.*, 1992), have been pioneered and evaluated as vehicles to deliver entrapped molecules to mammalian cells *in vitro* and *in vivo*, with a plethora of literature (e.g. Carafa *et al.*, 1998) citing the encapsulation of hydrophilic drugs; however limited attention has been committed to poorly water soluble drugs. To be effective as a carrier, a systematic investigation of the parameters that control the physico-chemical characteristics of liposomes containing bilayer-loaded drugs was required. Consequently the overall intentions of this research were to identify and understand the mechanistic processes associated in the development of liposomes that can be used as solubilisation agents. The choice of model drug candidates for formulation studies included drugs that varied in terms of physiochemical characteristics such as lipophilicity, nature of functional moieties and molecular weight; they were diazepam, ibuprofen, midazolam, phenytoin, propofol and rifampicin.

A systematic approach was undertaken beginning with optimisation work. Initial aims of the project were to investigate the techniques and methods that can be used to quantitatively describe drug encapsulation in MLV with their respective advantages and limitations examined. At this stage a method that offered the quickest throughput of formulations was selected as the central technique for analysis. Results obtained via the indirect method showed, that where sample is centrifuged to remove precipitated species and the supernatant extracted for analysis using UV, to be comparable to those obtained via more direct routes such as HPLC, with the former method rendering advantages of ease, speed, reproducibility, and consistent removal of precipitated MLV compared with HPLC. As a result, the indirect UV-based

procedure was used for all further analysis for bilayer drug quantification. Thereafter, this method was optimised, with drug recovery and centrifugation closely examined.

Subsequent preliminary investigations focused on the drug loading of ibuprofen within different MLV systems. Liposomes suffer some fundamental problems such as chemical instability and complications in mass production (Sinha *et al.*, 2002). Therefore, to eliminate such drawbacks, niosomes were evaluated as an alternative drug delivery vehicle since these carrier systems are prepared from non-ionic surfactants that are biodegradable, inexpensive and relatively stable (Sinha *et al.*, 2002; Yoshioka *et al.*, 1994). Initially both liposomal and niosomal formulations were screened for their drug-loading capacity: liposomal systems were shown to offer significantly higher ibuprofen loading and thereafter lipid-based systems were further investigated. Attempts were also made to establish the effect of lamellarity of the vesicle on drug encapsulation. Data collected suggested that lipid: drug ratio is probably more essential than the morphology of the preparation. Therefore, for all further liposomal studies, the substantially larger MLV (as opposed to SUV) were formulated and assessed for solubilisation of poorly water-soluble drugs.

Within this study one of our aims was to quantitatively and qualitatively investigate the effect of cholesterol concentration on stability of liposomes prepared to deliver solubilised lipophilic drugs as it is established that the presence of cholesterol provides a degree of physical bilayer stability in relative terms. Given the key role cholesterol is known to play within the stability of bilayer vesicles (Devaraj *et al.*, 2002, Gallova *et al.*, 2004 and Puglisi *et al.*, 1995), the optimum cholesterol content in terms of drug loading and release of poorly soluble drugs was then investigated.

Studies showed that increasing cholesterol content also reduced drug-encapsulation capabilities of liposomes. However, the release profiles of propofol from the various liposome formulations demonstrated that the increase in the molar ratio of cholesterol progressively decreased the release of propofol from the vesicles. This data suggested that there may be a competitive interaction between drug loading in the liposomal bilayer and cholesterol bilayer content.

The influence of the presence and concentration of cholesterol was assessed at the air/water interface using Langmuir monolayers where data collated revealed cholesterol inducing a 'condensing effect' in mixed phospholipid/cholesterol monolayers at the air-water interface, probably due to good bilayer interpacking, thereby suggesting a strong interaction between the two components. The Langmuir data possibly explained the decreased entrapment and cumulative release of drug with increase in cholesterol content. A further investigation using differential scanning calorimetry could examine any potential changes with inclusion of variable portions of cholesterol and drug, thereby aiding understanding of the possible competitive nature of the substances within the bilayer. Nevertheless, from these studies a concentration of 11 total molar % of cholesterol translated as the optimum concentration that balanced the three parameters, stability, rigidity and permeability, in order to achieve optimum encapsulation efficiency of a poorly water-soluble drug and thus formed the basis for all further formulations.

Attempts were made to prepare and characterise various liposome-based formulations to understand the factors responsible for the potential development of an efficient and stable liposomal formulation and finally to evaluate any potential toxicity concerns of

the liposome-based formulations. Results obtained suggest that the suitable choice of composition and surface charge can contribute to the design of liposomes suitable for acting as carriers for hydrophobic drugs for attempts at therapy. Investigating the effect of liposome composition on several low solubility drugs, drug loading was shown to be enhanced by adopting longer chain length lipids, cationic lipids and, decreasing drug molecular weight. Drug release was increased by using cationic lipids and lower molecular weight of drug; conversely, a reduction was noted when employing longer chain lipids thus supporting the rationale of longer chain lipids producing more stable liposomes, a theory also supported by results obtained via Langmuir studies- although it was revealed that stability is also dependent on geometric features associated with the lipid chain moiety. Although results showed that a near-fully charged bilayer increases the incorporation of a hydrophobic model drug, questions arose regarding the stability of using cationically-charged lipids since results from ESEM analysis advocated that a charged formulation is not as stable a formulation when weighed against its neutral counterpart and appears to be toxic to two types of cells (African green monkey kidney fibroblast (COS-7) cells and red blood cells)- both key obstacles in the development of pharmaceutical formulations.

As an alternative to the symmetrical lipid-based liposome formulation, investigations then turned towards the analysis of asymmetrical lipid-based formulations for the delivery of poorly water-soluble drug since many biological lipids are composed of asymmetric alkyl tail chains, where the tail moiety differs by their carbon number, presence and degree of saturation, or both (Dan, 2007). Therefore it was of interest to investigate the effect of geometry of phospholipid on hydrophobic solute encapsulation. Interestingly, a decrease in drug loading appeared to be induced when

asymmetrical phospholipids were used in place of symmetrical lipids as a result further stressing the importance of lipid geometry. Combining a symmetrical lipid with an asymmetrical derivative enhanced encapsulation of a hydrophobic drug while reducing that of another suggesting the importance of drug characteristics. Similar to symmetrical lipids, asymmetrical counterparts were found to be non-toxic to COS-7 and red blood cells.

The influence of drug characteristics on bilayer loading was examined. The investigation indicated a greater influence by molecular weight as opposed to lipophilicity (Log P) of drug in determining encapsulation rates since decreasing molecular weight resulted in enhancing drug loading within liposomes. The results indicated that the drug carried by liposomes may play a key role in liposome behaviour. To further understand the behaviour of drugs in the bilayer, differential scanning calorimetry could be applied to compare the thermal changes caused by each drug when incorporated in phospholipid bilayers.

Although numerous phospholipids for liposomes and several non-ionic surfactants for niosomes have undergone exploration in the formulation of vesicles, cholesterol is the sole substance used in the stabilisation of bilayer membranes. It was therefore apparent that an alternative ingredient was needed to offer another means of stabilising vesicles, thus forming the basis of the rationale behind the work that has been described in this instance. Recently, stable niosomes have successfully been formulated from fatty alcohols (Devaraj *et al.*, 2002). Therefore it was instructive to study the effect of these compounds on liposomal parameters. Liposomes were successfully formulated from fatty alcohols thereby showing potential for a novel

class of compounds to stabilise liposomal formulations- this satisfied one of the key aims of achieving an alternative liposomal stabiliser to cholesterol.

Results obtained revealed that liposomes containing tetradecanol (a fatty alcohol) within their formulation shared similar vesicle size, drug encapsulation, surface charge, and toxicity profiles as liposomes formulated with cholesterol, although the tetradecanol preparation appeared to release considerably more drug during stability studies. However, evidence obtained suggests that tetradecanol may have a reduced influence within the bilayer compared to cholesterol, manifesting as relatively decreased packing densities of phospholipid molecules by the former. This would suggest the cholesterol remains the main lipid of choice to be used in bilayer stabilisation.

Encouragingly, following the initial investigations presented herein, fatty alcohols present as promising substances for stabilising liposomal formulations. However, alcohol-based liposome formulations require further investigation in order to be taken forward as promising bilayer stabilisers for phospholipid-based liposome systems. In addition to Langmuir monolayer studies, differential scanning calorimetry (DSC) was also used to potentially explain the interaction of tetradecanol or cholesterol at the bilayer level by scrutinising thermal changes induced by both substances. The thermotropic phase behaviour of DSPC and cholesterol/tetradecanol was investigated using DSC. DSC scans showed typical results for mixtures of cholesterol with DSPC: broadening of the transition and lowering of the phase transition with increased molar fraction of the stabiliser as already shown by several research groups (e.g. Spink *et al.*, 1996). Performing the same experiments while substituting the cholesterol with

tetradecanol revealed that the lowering of the transition by the fatty alcohol was not as prominent as seen on scans produced for DSPC/cholesterol mixtures thereby suggesting that the fluidising effect commonly suggested for cholesterol (Ohtake *et al.*, 2005; Vilcheze *et al.*, 1996) is not as prominently induced by tetradecanol. This could further explain the considerably more drug release by the fatty alcohol preparation compared with its cholesterol-based analogue. However, the scans have not been presented here as, due to a malfunction in the associated computer hard-drive, the data yielded was lost.

To effectively deliver a formulation, the preparation must display stability. Investigations into an alternative assay for determining stability of liposomes prepared to deliver solubilised lipophilic drugs were performed. Using environmental scanning electron microscopy (ESEM) the real-time stability of liposomes during dehydration was followed. ESEM is a new technique for studying liposomes that can generate images of wet systems without the need for pre-preparation. ESEM was used to analyse the morphology and stability of liposomes to examine whether the technique has the potential to be a vehicle for assessing stability as per some promising published early data (Mohammed *et al.*, 2004; Perrie *et al.*, 2007; Vangala *et al.*, 2006). Investigations here indicated that the presence of drugs within the liposomal bilayer were able to enhance the stability of the bilayers against collapse under reduced hydration conditions. In addition the presence of charged lipids within the formulation under reduced hydration conditions compared with its neutral counterpart. However the applicability of using ESEM as a new method to investigate liposome stability appears less valid than first hoped since the results are often open to

varied interpretation and do not provide a robust set of data to support conclusions in some cases.

A review of literature indicated that by using appropriate fit models and analysis techniques, SANS has the potential to yield key information on the shape and size of the scattering bodies in the liposome mixtures. On this basis, neutron scattering was used to study if increase in lipid alkyl chain length and entrapment of poorly water soluble drugs within liposomes lead to changes in bilayer thickness. However, due to practical problems, data could not be obtained. Nevertheless, reviewed published data suggest that SANS can be used to assess bilayer mechanics. A possible modification of methodology could be using SUV in place of MLV.

In summary, key factors that can increase the solubilisation of a drug within liposomal bilayers are:

- Bilayer hydrophobic volume – employ longer chain lipids
- Charged surfactants – employ cationic lipids such as DSTAP
- Molecular size
- Symmetrical lipids
- Using ionic surfactants as opposed to non-ionic counterparts
- Combining a symmetrical phospholipid with an unsymmetrical one

Additionally, in summary, key factors that can enhance the stability of liposomal bilayers are:

- Bilayer hydrophobic volume – employ longer chain lipids

- Zwitterionic surfactants – employ neutral lipids such as DSPC as opposed to DSTAP
- Cholesterol
- Fatty alcohols
- Presence of drug

Overall the objectives of the outlined research plan have been met; essentially, a series of model poorly soluble drugs were used to determine the physicochemical characteristics and compositions of stable surfactant-based colloidal systems which promote hydrophobic drug loading and retention within the bilayer under various conditions in order to better design and engineer solubility enhancing liposomal systems tailored to the need of the drug and the route of administration. The liposomes were shown as a good delivery systems for poorly soluble drugs, and with careful formulation work- which depend greatly on the purpose of application, and deliberation of many physico-chemical parameters (as highlighted throughout)- coupled with their numerous advantages, can be suggested for passive tumour sites and inflammation via parenteral injection. The development of colloidal technology should be aided by past advancements, several of which have been expressed here, but with the realisation (as backed by results obtained here) the need to be both imaginative and systematic in the development of colloidal systems that hopefully will bring about more colloidal-based products on the market.

Further investigations could include Langmuir work on the effects of drug in phospholipid bilayers that have been stabilised by tetradecanol and compared with analogous formulations containing cholesterol. Moreover, other techniques such as

NMR, fluorescence anisotropy and X-ray powder diffraction could further substantiate the location and interaction of the stabilisers and drugs within the bilayer. In future, these liposomes (and other formulations examined) could be administered *in vivo*.

Future work needs to be focused on using formulation knowledge obtained here using model drug candidates and from past endeavours to attempt to improve efficacy of key problematic drugs in treatment of diseases. It is apparent that pharmaceutical research and scientists are responding very well to the challenge of poorly soluble drugs and that we may expect sustained progress in the improvement of solubility in the foreseeable future.

References

- Abou-Jaoude, M.M., Najm, R., Shaheen, J., Nawfal, N., Abboud, S., AlHabash, M., Darwish, M., Mulhem, A., Ojeh, A., Almawi, W.Y., 2005. Tacrolimus (FK506) versus cyclosporine microemulsion (neoral) as maintenance immunosuppression therapy in kidney transplant recipients. *Transplantation Proceedings* **37**(7), 3025-3028.
- Agarwal, R., Katare, O.P., Vyas, S.P., 2001. Preparation and in vitro evaluation of liposomal/niosomal delivery systems for antipsoriatic drug dithranol. *International Journal of Pharmaceutics* **228**, 43-52.
- Ahl, P.L., Chen, L., Perkins, W.R., Minchey, S.R., Boni, L.T., Taraschi, T.F., Janoff, A.S., 1994. Interdigitation-fusion: a new method for producing lipid vesicles of high internal volume. *Biochimica et Biophysica Acta* **1195**, 237-244.
- Allison, A.G., and Gregoriadis, G., 1974. Liposomes as immunological adjuvants. *Nature* **252**(252).
- Almasy, L., Szekely, N.K., Len, A., Muzsnay, C.S., Kiraly, K.N., 2005. Small-angle neutron scattering study of aqueous solutions of 1,7-heptanediol. *Journal of Molecular Liquids* **118**, 141-143.
- Alonso, J.M., Llacer, C., Vila, A.O., Figueruelo, J.E., Molina, F.J., 1995. Effect of the osmotic conditions on the value of zeta potential of DMPC multilamellar liposomes. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **95**(1), 11-14.
- Alsarra, I.A., Bosela, A.A., Ahmed, S.M., Mahrous, G.M., 2005. Proniosomes as a drug carrier for transdermal delivery of ketorolac. *International Journal of Pharmaceutics and Biopharmaceutics* **59**, 485-490.
- Altomare, C., Trapani, G., Latrofa, A., Serra, M., Sanna, E., Biggio, G., Liso, G., 2003. Highly water-soluble derivatives of the anesthetic agent propofol: in vitro and in vivo evaluation of cyclic amino acid esters. *European Journal of Pharmaceutical Sciences* **20**, 17-26.
- Andersin, R. and Tammilehto, S., 1995. Photochemical decomposition of midazolam IV. Study of pH-dependent stability by high-performance liquid chromatography. *International Journal of Pharmaceutics* **123**, 229-235.
- Ascarateil, S. and Dupuis, L., 2005. Surfactants in vaccine adjuvants: description and perspectives. *Vaccine* **24**, S83-S85.
- Avdeef, A., Kansy, M., Bendels, S., Tsinman, K., 2008. Absorption-excipient-pH classification gradient maps: Sparingly soluble drugs and the pH partition hypothesis. *European Journal of Pharmaceutical Sciences* **33**, 24-91.

Balasubramaniam, S.V. and Straubinger, R.M., 1994. Taxol-lipid interactions: taxol-dependent effects on the physical properties of model membranes. *Biochemistry* **33**, 8941-8947.

Balgavy, P., Dubnickova, M., Kucerka, N., Kiselev, M.A., Yaradaikin, S.P., Uhrikova, D., 2001. Bilayer thickness and lipid interface area in unilamellar extruded 1,2-diacylphosphatidylcholine liposomes: a small-angle neutron scattering study. *Biochimica et Biophysica Acta* **1512**, 40-52.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology* **13**, 325-328.

Barich, D.H., Munson, E.J., Zell, M.T., 2005. 'Physiochemical properties, formulation, and drug delivery', in Wang, B., Siahaan, T., Soltero, R.A., (eds.) *Drug Delivery: Principles and Applications*, John Wiley & Sons, Inc., Hoboken, New Jersey, 57-71.

Barillaro, V., Bertholet, P., de Hassonville, S.H., 2004. Effect of acidic ternary compounds on the formation of miconazole/cyclodextrin inclusion complexes by means of supercritical carbon dioxide. *Journal of Pharmaceutics* **7**(3), 378-388.

Barnes, G.T. and Gentle, I.R., 2005. *Interfacial Science: An Introduction*, Oxford University Press Inc., New York.

Batzri, S. and Korn, E.D., 1973. Single bilayer liposomes prepared without sonication. *Biochimica et Biophysica Acta* **298**, 1015-1019.

Bellott, R., Pouna, P., Robert, J., 2001. Separation and determination of liposomal and non-liposomal daunorubicin from the plasma of patients treated with Daunoxome. *Journal of Chromatography B: Biomedical Sciences and Applications* **757**(2), 257-267.

Bennati, C.R., Epand, R.M., Lamy, M.T., 2007. Low cholesterol solubility in DODAB liposomes. *Chemistry and Physics of Lipids* **145**, 27-36.

Bermudez, M., Martinez, E., Mora, M., Sagrista, M.L., de Madariaga, M.A., 1999. Molecular and physiochemical aspects of the interactions of the tuberculostatics ofloxacin and rifampicin with liposomal bilayers: a ³¹P-NMR and DSC study. *Colloids and Surfaces A: Physiochemical and Engineering Aspects* **158**, 59-66.

Bhatia, A., 2004. Tamoxifen in topical liposomes: development, characterization and in-vitro evaluation. *Journal of Pharmacy and Pharmaceutical Sciences* **7**(2), 252-259.

Biju, S.S., Sushama, T., Mishra, P.R., Khar, R.K., 2006. Vesicular systems: An overview. *Indian Journal of Pharmaceutical Sciences* **68**(2), 141-153.

- Boija, E., Lundquist, A., Pla, J.J.M., Engvall, C., Lundahl, P., 2004. Effects of ions and detergents in drug partition chromatography on liposomes. *Journal of Chromatography* **1030**(1-2), 273-278.
- Borenfreund, E., Babich, H., Martin-Alguacil, N., 1988. Comparisons of two *in vitro* cytotoxicity assays— The neutral red (NR) and tetrazolium MTT tests. *Toxicology in Vitro* **2**(1), 1-6.
- Brzustowicz, M.R., Cherezo, V., Zerouga, M., Caffrey, M., Stillwell, W., Wassall, S.R., 2002. Controlling membrane cholesterol content. A role for polyunsaturated (docosahexaenoate) phospholipids. *Biochemistry* **41**, 12509-12519.
- Buckton, G. (ed.), 1995. *Interfacial Phenomena in Drug Delivery and Targeting*, Harwood Academic Publishers, Chur, Switzerland.
- Budai, L., Hajdu, M., Budai, M., Grof, P., Beni, S., Noszal, B., Klebovich, I., Antal, I., 2007. Gels and liposomes in optimized ocular drug delivery: Studies on ciprofloxacin formulations. *International Journal of Pharmaceutics* **343**, 34-40.
- Bustamante, P., Romero, S., Pena, A., Escalera, B., Reillo, A., 1998. Nonlinear enthalpy-entropy compensation for the solubility of drugs in solvent mixtures: paracetamol, acetanilide and nalidixic acid in dioxane-water. *Journal of Pharmaceutical Sciences* **87**, 1590-1596.
- Cairns, D., 2003. *Essentials of Pharmaceutical Chemistry*, 2nd edn., Pharmaceutical Press, London, UK.
- Cao, Y. and Li, H., 2002. Interfacial activity of a novel family of polymeric surfactants. *European Polymer Journal* **38**, 1457-1463.
- Cao, Y., Chen, J., Wang, Y., Liang, J., Chen, L., Lu, Y., 2005. HPLC/UV analysis of chlorfenapyr residues in cabbage and soil to study the dynamics of different formulations. *Science of the Total Environment* **350**, 38-46.
- Carafa, M., Santucci, E., Alhaique, F., Coviello, T., Murtas, E., Riccieri, F.M., Lucania, G., Torrisi, M.R., 1998. Preparation and properties of new unilamellar non-ionic/ionic surfactant vesicles. *International Journal of Pharmaceutics* **160**, 51-59.
- Cardenas, M., Nylander, T., Jonsson, B., Lindman, B., 2005. The interaction between DNA and cationic lipid films at the air-water interface. *Journal of Colloid and Interface Science* **286**, 166-175.
- Casals, E., Gallardo, M., Estelrich, J., 1996. Factors influencing the encapsulation of thioguanine in DRV liposomes. *International Journal of Pharmaceutics* **143**, 171-177.

- Castelli, F., Sarpietro, M.G., Rocco, F., Ceruti, M., Cattel, L., 2007. Interaction of lipophilic gemcitabine prodrugs with biomembrane models studied by Langmuir-Blodgett technique. *Journal of Colloid and Interface Science* **313**, 363-368.
- Chen, R., Yue, Z., Eccleston, M.E., Williams, S., Slater, N.K.H., 2005. Modulation of cell membrane disruption by pH- responsive pseudo-peptides through grafting with hydrophilic side chains. *Journal of Controlled Release* **108**, 63-72.
- Cortesi, R., Esposito, E., Maietti, A., Menegatti, E., Nastruzzi, C., 1997. Formulation study for the antitumor drug camptothecin: liposomes, micellar solutions and a microemulsion. *International Journal of Pharmaceutics* **159**, 95-103.
- Cortesi, R. and Nastruzzi, C., 1999. Liposomes, micelles and microemulsions as new delivery systems for cytotoxic alkaloids. *PSTT* **2**(7), 288-298.
- Coster, H.G.L. and Chilcott, T.C., 1996. Role of fixed charges in biological membranes. *Progress in Biophysics and Molecular Biology* **65**(1), 166-166.
- Csiszar, A., Klumpp, E., Bota, A., Szegedi, K., 2003. Effect of 2,4-dichlorophenol on DPPC/water liposomes studied by X-ray and freeze-fracture electron microscopy. *Chemistry and Physics of Lipids* **126**, 155-166.
- Dan, 2007. Lipid tail chain asymmetry and the strength of membrane-induced interactions between membrane proteins. *Biochimica et Biophysica Acta* **1768**, 2393-2399.
- Davies, J.T. and Rideal, E.K., 1963. *Interfacial Phenomena*, Academic Press Inc, New York, USA
- Delaney, J.S., 2005. Predicting aqueous solubility from structure. *DDT* **10**(4), 289-295.
- Demel, R.A., Geurts van Kessel, W.S.M., Van Deenen, L.L.M., 1972. The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. *Biochimica Biophysica Acta* **266**, 26-40.
- Deol, P. and Khuller, G.K., 1997. Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. *Biochimica et Biophysica Acta* **1334**, 161-172.
- Devaraj, G.N., Parakh, S.R., Devraj, R., Apte, S.S., Ramesh Rao, B., Rambhu, D., 2002. Release studies on niosomes containing fatty alcohols as bilayer stabilisers of cholesterol. *Journal of Colloid and Interface Science* **251**, 360-365.
- Di Profio, P., Arca, S., Germani, R., Savelli, G., 2005. Surfactant promoting effects on clathrate hydrate formation: Are micelles really involved?. *Chemical Engineering Science* **60**(15), 4141-4145.

- Dong, X. and Mumper, R.J., 2006. The metabolism of fatty alcohols in lipid nanoparticles by alcohol dehydrogenase. *Drug Development and Industrial Pharmacy* **32**(8), 973-980.
- Dougherty, D.A. and Stauffer, D.A., 1990. Acetylcholine binding by a synthetic receptor: implications for biological recognition. *Science* **250**, 1558-1560.
- Dragnevski, K.I. and Donald, A.M., 2008. An environmental scanning electron microscopy examination of the film formation mechanism of novel acrylic latex. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **317**, 551-556.
- Dressman, J., 2007. Drug solubility: how to measure it, how to improve it. *Advanced Drug Delivery Reviews* **59**, 531-532.
- Drummond, D.C., Meyer, O., Hong, K., Kirpotin, D.B., Papahadjopoulos, D., 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *The American Society for Pharmacology and Experimental Therapeutics* **51**(4), 691-743.
- du Plessis, J., Ramachandran, C., Weiner, N., Muller, D.G., 1996. The influence of lipid composition and lamellarity of liposomes on the physical stability of liposomes upon storage. *International Journal of Pharmaceutics* **127**, 273-278.
- Dynarowicz-Latka, P., Dhanabalan, A., Oliveira Jr., O.N., 2001. Modern physicochemical research on Langmuir monolayers. *Advances in Colloid and Interface Science* **91**, 221-293.
- Dynarowicz-Latka, P. and Hac-Wydro, K., 2004. Interactions between phosphatidylcholines and cholesterol in monolayers at the air/water interface. *Colloids and Surfaces B: Biointerfaces* **37**, 21-25.
- Ebrahim, S., Gholam, A., Peyman, A., Lee, P.J., 2005. Applications of liposomes in ophthalmology. *Survey of Ophthalmology* **50**(2), 167-182.
- ECVAM. European Centre for the Validation of Alternative Methods, 192. INVITOX Protocol N° 37 Red blood cells test system.
- Edwards, K.A. and Baeumner, A.J., 2006. Analysis of liposomes. *Talanta* **68**, 1432-1441.
- Fahr, A., van Hoogevest, P., May, S., Bergstrand, N., Leigh, M.L.S., 2005. Transfer of lipophilic drugs between liposomal membranes and biological interfaces: consequences for drug delivery. *European Journal of Pharmaceutical Sciences* **26**, 251-265.
- Faller, B. and Ertl, P., 2007. Computational approaches to determine drug solubility. *Advanced Drug Delivery Reviews* **59**, 533-545.

- Fang, J-Y., Hong, C-T., Chiu, W-T., Wang, Y-Y., 2001. Effect of liposomes and niosomes on skin permeation of enoxacin. *International Journal of Pharmaceutics* **219** (1-2), 61-72.
- Farrar, H., Letzig, L., Gill, M., 2002. Validation of a liquid chromatographic method for the determination of ibuprofen in human plasma. *Journal of Chromatography B* **780**, 341-348.
- Fatouros, D.G. and Antimisiaris, S.G., 2002. Effect of amphiphilic drugs on the stability and zeta-potential of their liposome formulations: a study with prednisolone, diazepam, and griseofulvin. *Journal of Colloid and Interface Science* **251**, 271-277.
- Faucon, J-F., Bonmatin, J-M., Dufourcq, J., Dufourc, E.J., 1995. Acyl chain length dependence in the stability of melittin-phosphatidylcholine complexes. A light scattering and ^{31}P -NMR study. *Biochimica et Biophysica Acta* **1234**, 235-243.
- FDA/CDER, 2006. *The Biopharmaceutics Classification System (BCS) guidance*, FDA/Centre for Drug Evaluation and Research, Maryland, USA, Viewed 11 June 2008, Available from: http://www.fda.gov/cder/OPS/BCS_guidance.htm
- Felnerova, D., Viret, J-F., Gluck, R., Moser, C., 2004. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. *Current Opinion in Biotechnology* **15**, 518-529.
- Finlay, W.H. and Wong, J.P., 1998. Regional lung deposition of nebulized liposome-encapsulated Ciprofloxacin. *International Journal of Pharmaceutics* **167**, 121-127.
- Florence, A., and Attwood, D., 1998. *Physicochemical Principles of Pharmacy*, 3rd edn., Macmillan Press, London, UK.
- Fresta, M., Spadaro, A., Cerniglia, G., Ropero, I.M., Puglisi, G., 1995. Intracellular accumulation of ofloxacin-loaded liposomes in human synovial fibroblasts. *Antimicrobial Agents and Chemotherapy* **39**(6), 1372-1375.
- Gaines, G.L, 1966. *Insoluble Monolayers at Liquid-Gas Interfaces*, John Wiley & Sons Inc., New York.
- Gallova, J., Uhrikova, D., Hanulova, M., Teixeira, J., Balgavy, P., 2004. Bilayer thickness in unilamellar extruded 1,2-dimyristoleoyl and 1,2-dierucoyl phosphatidylcholine vesicles: SANS contrast variation study of cholesterol effect. *Colloids and Surfaces B: Biointerfaces* **38**, 11-14.

- Garau, C., Frontera, A., Quinonero, D., Ballester, P., Costa, A., Deya, P.M., 2004. Cation- π vs anion- π interactions: a complete π -orbital analysis. *Chemical Physics Letters* **399**, 220-225.
- Gilbert, R.J.C., Heenan, R.K., Timmins, P.A., Gingles, N.A., Mitchell, T.A., Rowe, A.J., Rossjohn, J., Parker, M.W., Andrew, P.W., Byron, O., 1999. Studies on the structure and mechanism of a bacterial protein toxin by analytical ultracentrifugation and small-angle neutron scattering. *Journal of Molecular Biology* **293**, 1145-1160.
- Goerg, H.C., Coutinho, K., Canuto, S., 2005. A look inside the cavity of hydrated α -cyclodextrin: a computer simulation study. *Chemical Physics Letters* **413**, 16-21.
- Gopinath, D., Ravi, D., Rao, B.R., Apte, S.S., Renuka, D., Rambhau, D., 2004. Ascorbyl palmitate vesicles (Aspasomes): formation, characterization and applications. *International Journal of Pharmaceutics* **271**, 95-113.
- Gregoriadis, G., Leathwood, P.D., Ryman, B. E., 1971. *FEBS Letters* **14**(2): 95-99.
- Gregoriadis, G., 1973. Drug entrapment in liposomes. *FEBS Letters* **36**(3), 292-296.
- Gregoriadis, G., Wills, E.J., Swain, C.P., Tavill, A.S., 1974. Drug-carrier potential of liposomes in cancer chemotherapy. *Lancet* **I**, 1313-1316.
- Gregoriadis, G. and Davis, C., 1979. Stability of liposomes *in vivo* and *in vitro* is promoted by their cholesterol content and the presence of blood cells. *Biochemical and Biophysical Research Communications* **89**(4), 1287-1293.
- Gregoriadis, G. and Senior, J., 1980. The phospholipids component of small unilamellar liposomes controls the rate of clearance of entrapped solutes from the circulation. *FEBS Letters* **119**(1), 43-46.
- Gregoriadis, G., 1985. Liposomes for drugs and vaccines. *Trends in Biotechnology* **3**(9), 235-241.
- Gregoriadis, G., McCormack, B., Obrenovic, M., Saffie, R., Zadi, B., Perrie, Y., 1999. Vaccine entrapment in liposomes. *Methods* **19**, 156-162.
- Gregoriadis, G., McCormack, B., Obrenovic, M., Perrie, Y., Yang, J-C., 2000. 'Genetic vaccines: A role for liposomes', in Gregoriadis, G. and McCormack, B. (eds.), *Targeting of Drugs: Strategies for Gene Constructs and Delivery*, IOS Press, Amsterdam, Netherlands, 92-101.
- Guo, W., Ahmad, A., Khan, S., Dahhani, F., Wang, Y.F., Ahmad, I., 2003. Determination by liquid chromatography with fluorescence detection of total 7-ethyl-10-hydroxycamptothecin (SN-38) in beagle dog plasma after intravenous administration of liposome-based SN-38 (LE-SN38). *Journal of Chromatography B* **791**, 85-92.

Gursoy, A., Kut, E., Ozkirimli, S., 2004. Co-encapsulation of isoniazid and rifampicin in liposomes and characterization of liposomes by derivative spectroscopy. *International Journal of Pharmaceutics* **271**, 115-123.

Hac-Wydro, K. and Wydro, P., 2007. The influence of fatty acids on model cholesterol/phospholipid membranes. *Chemistry and Physics of Lipids* **150**, 66-81.

Hac-Wydro, K., Wydro, P., Jagoda, A., Kapusta, J., 2007. The study on the interaction between phytosterols and phospholipids in model membranes. *Chemistry and Physics of Lipids* **150**, 22-34.

Hadgraft, J., Peck, J., Williams, D.G., Pugh, W.J., Allan, G., 1996. Mechanisms of action of skin permeation enhancers/retarders: Azone and analogues. *International Journal of Pharmaceutics* **141**, 17-25.

Hamada, F., Higuchi, Y., Kondo, Y., Kabuto, C., Iki, N., 2006. Supramolecular assembly based on π - π stacking π -cation interactions between thiacalix[6]arene and DMF. *Tetrahedron Letters* **47**, 5591-5593.

Harvey, R.D., Heenan, R.K., Barlow, D.J., Lawrence, M.J., 2005. The effect of electrolyte on the morphology of vesicles composed of the dialkyl polyoxyethylene ether surfactant 2C₁₈E₁₂. *Chemistry and Physics of Lipids* **133**, 27-36.

Harvey, R.D., Barlow, D.J., Drake, A.F., Kudsiova, L., Lawrence, M.J., Brain, A.P.R., Heenan, R.K., 2007. The effect of electrolyte on the encapsulation efficiency of vesicles formed by the nonionic surfactant, 2C₁₈E₁₂. *Journal of Colloid and Interface Science* **315**, 648-661.

Hathout, R.M., Mansour, S., Mortada, N.D., Guinedi, A.S., 2007. Liposomes as an ocular delivery system for acetazolamide: in vitro and in vivo studies. *AAPS PharmSciTech* **8**(1), Article 1.

Heurtault, B., Saulnier, P., Pech, B., Proust, J-E., Benoit, J-P., 2003. Physico-chemical stability of colloidal lipid particles. *Biomaterials* **24**, 4283-4300.

Hughes, G.A., 2005. Nanostructure-mediated drug delivery. *Nanomedicine: Nanotechnology, Biology, and Medicine* **1**, 22-30.

Ickenstein, L.M., Sandstrom, M.C., Mayer, L.D., Edwards, K., 2006. Effects of phospholipid hydrolysis on the aggregate structure in DPPC/DSPE-PEG₂₀₀₀ liposome preparations after gel to liquid crystalline phase transition. *Biochimica et Biophysica Acta* **1758**, 171-180.

Ishida, T., Takanashi, Y., Doi, H., Yamamoto, I., Kiwada, H., 2002. Encapsulation of an antivasospastic drug, fasudil, into liposomes, and in vitro stability of the fasudil-loaded

liposomes. *International Journal of Pharmaceutics* **232**, 59-57.

Ismail, A.A., Gouda, M.W., Motawi, M.M., 1970. Micellar solubilisation of barbiturates I: Solubilities of certain barbiturates in polysorbates of varying hydrophobic chain length. *Journal of Pharmaceutical Sciences* **59**, 220-224.

Israelachvili, J.N., Marcelja, S., Horn, R.G., 1980. Physical principles of membrane organization. *Quarterly Reviews of Biophysics* **13**(2), 121-200.

Kaganer, V.M., Mohwald, H., Dutta, P., 1999. Structure and phase transitions in Langmuir monolayers. *Reviews of Modern Physics* **71**(3), 779-819.

Kapitza, S.B., Michel, B.R., van Hoogevest, P., Leigh M.L.S., Imanidis, G., 2007. Absorption of poorly water soluble drugs subject to apical efflux using phospholipids as solubilizers in the Caco-2 cell model. *European Journal of Pharmaceutics and Biopharmaceutics* **66**, 146-158.

Khadikar, P.V., Agrawal, V.K., Karmarkar, S., 2002. Prediction of lipophilicity of polyacenes using quantitative structure-activity relationships. *Bioorganic & Medicinal Chemistry* **10**, 3499-3507.

Khatun, M., Islam, S.M.A., Akter, P., Quadir, M.A., Reza, M.S., 2004. Controlled release of naproxen sodium from Eudragit RS 100 transdermal film. *Dhaka University Journal of Pharmaceutical Sciences* **3**(1-2).

Kheirulomoom, A., Satpathy, G.R., Torok, Z., Banerjee, M., Bali, R., Novaes, R.C., Little, E., Manning, D.M., Dwyre, D.M., Tablin, F., Crowe, J.H., Tsvetkova, N.M., 2005. Phospholipid vesicles increase the survival of freeze-dried human red blood cells. *Cryobiology* **51**, 290-305.

Kim, C-J, 2004. *Advanced Pharmaceutics: Physicochemical Principles*, CRC Press LLC, Boca Raton, Florida, 113-191.

Kim, J-H., Kim, Y-S., Park, K., Lee, S., Nam, H.Y., Min, K.H., Jo, H.G., Park, J.H., Choi, K., Jeong, S.Y., Park, R-W., Kim, I-S., Kim, K., Kwon, I.C., 2008. Antitumor efficacy of cisplatin-loaded glycol chitosan nanoparticles in tumor-bearing mice. *Journal of Controlled Release* **127**(1), 41-49.

Kirby, C., Clarke, J., Gregoriadis, G., 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *Biochemical Journal* **186**, 591-598.

Kirby, C. and Gregoriadis, G., 1981. Plasma-induced release of solutes from small unilamellar liposomes is associated with pore formation in the bilayers. *Biochemical Journal* **199**, 251-254.

Kirby, C. and Gregoriadis, G., 1983. The effect of lipid composition of small unilamellar liposomes containing melphalan and vincristine on drug clearance after injection into mice. *Biochemical Pharmacology* **32**(4), 609-615.

Kirby, C.J. and Gregoriadis, G., 1984. Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. *Bio/Technology* November 1984, 979-984.

Kirby, C.J. and Gregoriadis, G., 1999. 'Liposomes' in Mathiowitz, E. (ed.), *Encyclopaedia of Controlled Drug Delivery*, John Wiley & Sons, Inc., New York, USA, 461-492.

Kirby, D., 2007. 'Formulation and characterisation of an effective particulate delivery vehicle for the novel sub-unit vaccine antigen, Ag85B-ESAT-6', PhD thesis. Medicines Research Unit, Aston University, Birmingham, UK.

Kobayashi, M., Sada, N., Sugawara, M., Iseki, K., Miyazaki, K., 2001. Development of a new system for prediction of drug absorption that takes into account drug dissolution and pH change in the gastro-intestinal tract. *International Journal of Pharmaceutics* **221**(1-2), 87-94.

Korchowiec, B., Paluch, M., Corvis, Y., Rogalska, E., 2006. A Langmuir film approach to elucidating interactions in lipid membranes: 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine/cholesterol/metal cation systems. *Chemistry and Physics of Lipids* **144**, 127-136.

Kragh-Hansen, U., le Maire, M., Moller, J.V., 1998. The mechanism of detergent solubilization of liposomes and protein-containing membranes. *Biophysical Journal* **75**(6), 2932-2946.

Lagarto, A., Vega, R., Vega, Y., Guerra, I., Gonzalez, R., 2006. Comparative study of red blood cell method in rat and calves blood as alternatives of Draize eye irritation test. *Toxicology in Vitro* **20**, 529-533.

Lancelot, E. and Grauby-Heywang, C., 2007. Comparison of the interaction of dihydrocholesterol and cholesterol with sphingolipid or phospholipid Langmuir monolayers. *Colloids and Surfaces B: Biointerfaces* **59**, 81-86.

Lappalainen, K., Jaaskelainen, I., Syrjanen, K., Urtti, A., Syrjanen, S., 1994. Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharmaceutical Research* **11**, 1127-1131.

Latrofa, A., Trapani, G., Franco, M., Serra, M., Miggironi, M., Fanizzi, F.P., Cutrignelli, A., Liso, G., 2001. Complexation of phenytoin with some hydrophilic cyclodextrins: effect on aqueous solubility, dissolution rate, and anticonvulsant activity in mice. *European Journal of Pharmaceutics and Biopharmaceutics* **52**, 65-73.

- Lawrence, M.J. and Barlow, D.J., 2006. 'Drug Delivery Systems: Neutron Scattering Studies' in Swarbrick, J. (ed.), *Encyclopedia of Pharmaceutical Technology*, 3rd edn., Informa Healthcare, New York, USA, viewed 18 August 2008, available from <<http://www.informaworld.com/smpp/1779139984-72238903/content~content=a758578175~db=refwork~order=title>>
- Lee, A.J., King, J.R., Hibberd, S., 1998. Mathematical modelling of the release of drug from porous, nonswelling transdermal drug-delivery devices. *IMA Journal of Mathematics Applied in Medicine & Biology* **15**, 135-163.
- Li, J.B., Kragel, J., Makievski, A.V., Fainermann, V.B., Miller, R., Mohwald, H., 1998. A study of mixed phospholipid/ β -casein monolayers at the water/air surface. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **142**, 355-360.
- Li, L.C. and Tian, Y., 2006, 'Zeta potential' in Swarbrick, J. and Boylan, J.C. (eds.), *Encyclopedia of Pharmaceutical Technology*, 2nd edn., Marcel Dekker, New York, USA, 4117-4128.
- Lin, J.M. and Yamada, M., 2003. Microheterogeneous systems of micelles and microemulsions as reaction media in chemiluminescent analysis. *Trends in Analytical Chemistry* **22**(2), 99-107.
- Lincopan, N., Borelli, P., Fock, R., Mamizuka, E.M., Carmona-Ribeiro, A.M., 2006. Toxicity of an effective amphotericin B formulation at high cationic lipid to drug molar ratio. *Experimental and Toxicologic Pathology* **58**, 175-183.
- Lipinski, C.A., 2000. Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological and Toxicological Methods* **44**, 235-249.
- Liu, X-Y., Yang, Q., Kamo, N., Miyake, J., 2001. Effect of liposome type and membrane fluidity on drug-membrane partitioning analyzed by immobilized liposome chromatography. *Journal of Chromatography A* **913**, 123-131.
- Liu, J. and Yang, L., 2006. Effect of cholesterol on DMPC phospholipid membranes and QSAR model construction in membrane-interaction QSAR study through molecular dynamics simulation. *Bioorganic & Medicinal Chemistry* **14**, 2225-2234.
- Lo, Y-L., Tsai, J-C., Kuo, J-H., 2004. Liposomes and disaccharides as carriers in spray-dried powder formulations of superoxide dismutase. *Journal of Controlled Release* **94**, 259-272.
- Loftsson, T., Hreinsdottir, D., Masson, M., 2005. Evaluation of cyclodextrin solubilisation of drugs. *International Journal of Pharmaceutics* **302**, 18-28.
- Loomis, C.R., Shipley, G.G., Small, D.M., 1979. The phase behaviour of hydrated

cholesterol. *Journal of Lipid Research* **20**, 525-535.

Lukyanov, A.N. and Torchilin, V.P., 2004. Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. *Advanced Drug Delivery Reviews* **56**, 1273-1289.

Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. *Journal of Controlled Release* **114**, 100-109.

Ma, J.C. and Dougherty, D.A., 1997. The cation- π interaction. *Chemical Reviews* **97**, 1303-1324.

Magazu, S., Migliardo, F., Barreca, D., Bellocob, E., Lagana, G., 2008. Neutron scattering study on the interaction between polyethylene glycol and lysozyme. *Physica B* **403**, 2408-2412.

Malmsten, M., Lindstrom, A-L., Warnheim, T., 1996. Electrostatic effects on interfacial film formation in emulsion systems. *Journal of Colloid and Interface Science* **179**, 537-543.

Manosroi, A., Wongtrakul, P., Manosroi, J., Sakai, H., Sugawara, F., Yuasa, M., Abe, M., 2003. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. *Colloids and Surfaces B: Biointerfaces* **30**, 129-138.

Manosroi, A., Wongtrakul, P., Manosroi, J., Midorikawa, U., Hanyu, Y., Yuasa, M., Sugawara, F., Sakai, H., Abe, M., 2005. The entrapment of kojic oleate in bilayer vesicles. *International Journal of Pharmaceutics* **298**, 13-25.

Martin, A.N., Bustamante, P., Chun, A.H., 1993. *Physical Pharmacy*, Lippincott, Williams & Wilkins, Philadelphia, USA.

Massing, U. and Fuxias, S., 2000. Liposoamal formulations of anticancer drugs: selectivity and effectiveness. *Drug Resistance Updates* **3**, 171-177.

Marikovsky, Y. and Danon, D., 1969. Electron microscope analysis of young and old red blood cells stained with colloidal iron for surface charge evaluation. *The Journal of Cell Biology* **43**(1), 1-7.

Mathews, R.G., Stokes, D.J., Thiel, B.L., Donald, A.M., 1999. 'Contrast formation in the Environmental Scanning Electron Microscope- imaging liquid-liquid systems', in Keily, C.J. (ed.), *Electron Microscopy and Analysis 1999* **161**, Institute of Physics Conference Series, CRC Press, London, UK, 95-98.

Matsuki, H., Miyazaki, E., Sakano, F., Tamai, N., Kaneshina, S., 2007. Thermotropic and barotropic phase transitions in bilayer membranes of ether-linked phospholipids with varying alkyl chain lengths. *Biochimica et Biophysica Acta* **1768**, 479-489.

Matsamura, Y. and Maeda, H., 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanisms of tumorotropic accumulation of protein and the antitumor agent SMANCS. *Cancer Research* **46**, 6387-6392.

Maurer, N., Wong, K.F., Stark, H., Louie, L., McIntosh, D., Wong, T., Scherrer, P., Semple, S.C., Cullis, P.R., 2001. Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. *Biophysical Journal* **80**(5), 2310-2326.

Minones Jr., J., Rey Gomez-Serranillos, I., Conde, O., Dynarowicz-Latka, P., Minones Trillo, J., 2006. The influence of subphase temperature on miltefosine-cholesterol mixed monolayers. *Journal of Colloid and Interface Science* **301**, 258-266.

Mohammed, A.R., Weston, N., Coombes, A.G.A., Fitzgerald, M., Perrie, Y., 2004. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *International Journal of Pharmaceutics* **285**, 23-34.

Mohammed, A.R., Bramwell, V.W., Coombes, A.G.A., Perrie, Y., 2006. Lyophilisation and sterilization of liposomal vaccines to produce stable and sterile products. *Methods* **40**(1), 30-38.

Mohammed, A.R., Coombes, A.G.A., Perrie, Y., 2007. Amino acids as cryoprotectants for liposomal delivery systems. *European Journal of Pharmaceutical Sciences* **30**(5), 406-413.

Momo, F., Fabris, S., Bindoli, A., Scutari, G., Stevanato, R., 2002. Different effects of propofol and nitrosopropofol on DMPC multilamellar liposomes. *Biophysical Chemistry* **95**, 145-155.

Momo, F., Fabris, S., Bindoli, A., Stevanato, R., 2005. Interaction of fluoxetine with phosphatidylcholine liposomes. *Biophysical Chemistry* **118**, 15-21.

Monzon, L.M.A. and Yudi, L.M., 2001. Voltammetric analysis of lipophilicity of benzodiazepine derivatives at the water/1,2-dichloroethane interface. *Journal of Electroanalytical Chemistry* **495**, 146-151.

Muller, H.M. and Keck, C.M., 2004. Challenges and solutions for the delivery of biotech drugs-a review of drug nanocrystal technology and lipid nanoparticles. *Journal of Biotechnology* **113**, 151-170.

Muller, M., Mackeben, S., Muller-Goymen, C.C., 2004. Physiochemical characterisation of liposomes with encapsulated local anaesthetics. *International Journal of Pharmaceutics* **274**, 139-148.

Nandi, I., Bateson, M., Bari, M., Joshi, H.N., 2003. Synergistic effect of PEG-400 and cyclodextrin to enhance solubility of progesterone. *AAPS PharmSciTech* **4**(1), Article 1,

1-5.

Nortd, S.P. and Clark, R.F., 1997. Midazolam: a review of therapeutic uses and toxicity. *The Journal of Emergency Medicine* **15**(3), 357-365.

Nounou, M., El-Khordagui, L.K., Khalafallah, N.A., Khalil, S.A., 2006. In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels. *Acta Pharmaceutica* **56**, 311-324.

Nawroth, T., Rusp, M., May, R.P., 2004. Magnetic liposomes and entrapping : time-resolved neutron scattering TR-SANS and electron microscopy. *Physica B* **350**, e635-e638.

O'Connor, K.M. and Corrigan, O.I., 2001. Comparison of the physicochemical properties of the *N*-(2-hydroxyethyl) pyrrolidine, diethylamine and sodium salt forms of diclofenac. *International Journal of Pharmaceutics* **222**, 281-293.

Ohtake, S., Schebor, C., Palecek, S.P., de Pablo, J.J., 2005. Phase behavior of freeze-dried phospholipids-cholesterol mixtures stabilized with trehalose. *Biochimica et Biophysica Acta* **1713**, 57-64.

Omran, A.A., Kitamura, K., Takegami, S., El-Sayed, A-A.Y., Abdel-Mottaleb, M., 2001. Determination of partition coefficients of diazepam and flurazepam between phosphatidylcholine bilayer vesicles and water by second derivative spectrophotometric method. *Journal of Pharmaceutical and Biomedical Analysis* **25**, 319-324.

Ong, J.T.H. and Manoukian, E., 1988. Micellar solubilization of timbesome acetate in aqueous and aqueous propylene glycol solutions of nonionic surfactants. *Pharmaceutical Research* **3**(11), 704-708.

Oussoren, C., Eling, W.M.C., Crommelin, D.J.A., Storm, G., Zuidema, J., 1998. The influence of the route of administration and liposome composition on the potential of liposomes to protect tissue against local toxicity of two antitumor drugs. *Biochimica et Biophysica Acta* **1369**, 159-172.

Ovcharova, G.D., Nacheva, R.N., Dimitrova, D.B., 1990. A potentiometric method of quantitative analysis of rifampicin. *Antibiotikii Khimioterapiya* **35**(2), 8-10.

Pasquardini, L., Lunelli, L., Vanzetti, L., Anderle, M., Pederzoli, C., 2008. Immobilization of cationic rifampicin-loaded liposomes on polystyrene for drug-delivery applications. *Colloids and Surfaces B: Biointerfaces* **62**, 265-272.

Pearce, J., 2006. *Quantitative UV analysis*, Science of Spectroscopy 2006, viewed 15 June 2008, <http://www.scienceofspectroscopy.info/edit/index.php?title=Quantitative_UV_Analysis>

- Pencer, J., Nieh, M-P., Harroun, T.A., Krueger, S., Adams, C., Katsaras, J., 2005. Bilayer thickness and thermal response of dimyristoylphosphatidylcholine unilamellar vesicles containing cholesterol, ergosterol and lanosterol: A small-angle neutron scattering study. *Biochimica et Biophysica Acta* **1720**, 84-91.
- Perrett, S. and Venkatesh, G., 2006. Enhancing the bioavailability of insoluble drug compounds. *Innovations in Pharmaceutical Technology* April 2006, 80-85.
- Perrie, Y. and Gregoriadis, G., 2000. Liposome-entrapped plasmid DNA: characterisation studies. *Biochimica et Biophysica Acta* **1475**, 125-132.
- Perrie, Y., Barralet, J.E., McNeil, S., Vangala, A., 2004. Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route. *International Journal of Pharmaceutics* **284**(1-2), 31-41.
- Perrie, Y., 2005. Liposome delivery of sparingly soluble drugs. *European Biopharmaceutical Review* Autumn, 90-97.
- Perrie, Y., Mohammed, A.R., Vangala, A., McNeil, S.E., 2007. Environmental Scanning Electron Microscopy offers real-time morphological analysis of liposomes and niosomes. *Journal of Liposome Research* **17**(1), 27-37.
- Perrut, M., Jung, J., Leboeuf, F., 2005. Enhancement of dissolution rate of poorly-soluble active ingredients by supercritical fluid processes. Part I: micronization of neat particles. *International Journal of Pharmaceutics* **288**, 3-10.
- Pouton, C.W., 2006. Formulation of poorly water-soluble drugs for oral administration: Physicochemical and physiological issues and the lipid formulation classification system. *European Journal of Pharmaceutical Sciences* **29**, 278-287.
- Prentis, R.A., Lis, Y., Walker, S.R., 1999. Pharmaceutical innovations in the seven UK-owned pharmaceutical companies (1964-1985). *British Journal of Clinical Pharmacology* **25**, 387-396.
- Proulx, M-E., Desormeaux, A., Marquis, J-F., Olivier, M., Bergeron, M.G., 2001. Treatment of visceral leishmaniasis with sterically stabilized liposomes containing camptothecin. *Antimicrobial Agents and Chemotherapy* **45**(9), 2623-2627.
- Puglisi, G., Fresta, M., Mazzone, G., Furneri, P.M., Tempera, G., 1995. Formulation parameters of fluoroquinolone-loaded liposomes and in vitro antimicrobial activity. *International Journal of Pharmaceutics* **118**, 65-76.
- Radlinski, A., 1999. Small-angle neutron scattering: A new technique to detect generated source rocks. *AGSO Research Newsletter* **31**, November.

- Rangel-Yagui, C.O., Pessoa Jr., A., Tavares, L.C., 2005. Micellar solubilization of drugs. *Journal of Pharmacy and Pharmaceutical Sciences* **8**(2), 147-163.
- Rautio, J., Kumpulainen, H., Heimbach, T., Oliyai, R., Oh, D., Jarvinen, T., Savolainen, J., 2008. Prodrugs: design and clinical applications. *Nature Reviews Drug Discovery* **7**(3), 255-270.
- Reimer, D., Eastman, S., Flowers, C., Boey, A., Redelmeier, T., Ouyang, C., 2005. Liposome formulations of sparingly soluble compounds: Liposome technology offers many advantages for formulation of sparingly soluble compounds. *Pharmaceutical Formulation & Quality* August/September 2005, 42-44.
- Remko, M. and von der Lieth, C-W., 2004. Theoretical study of gas-phase acidity, pKa, lipophilicity, and solubility of some biologically active sulfonamides. *Bioorganic & Medicinal Chemistry* **12**, 5395-5403.
- Rodier, E., Lochard, H., Sauceau, M., Letourneau, J.J., Freiss, B., Fages, J., 2005. A three step supercritical process to improve the dissolution rate of eflucimibe. *European Journal of Pharmaceutical Sciences* **26**, 184-193.
- Rogerson, A., Cummings, M.J., Florence, A.T., 1987. Adriamycin-loaded niosomes: drug entrapment, stability and release. *Journal of Microencapsulation* **4**, 321-328.
- Rong Liu, Cannon, J.C., Li, Y., 2000. 'Liposomes in solubilisation' in Rong Liu (ed.), *Water Insoluble Formulations*, 2nd edn., CRC Press, Boca Raton, Florida, USA, 355-404.
- Saarinen-Savolainen, P., Jarvinen, T., Taipale, H., Urtti, A., 1997. Method for evaluating drug release from liposomes in sink conditions. *International Journal of Pharmaceutics* **159**, 27-33.
- Saitoh, T., Hattori, K., Hiraide, M., 2005. Aerosol-OT micelles in Sephadex gels for concentrating metal-dithizone chelates from water. *Analytica Chimica Acta* **543**, 25-30.
- Santucci, E., Carafa, M., Coviello, T., Murtas, E., Ricciari, F.M., Alhaique, F., Modesti, A., Modica, A., 1996. Vesicles from polysorbate 20 and cholesterol. A simple preparation and characterisation. *STP Pharma Sciences* **6**, 29-32.
- Scriba, K.E. and Lambert, D.M., 1997. Bioavailability of phenytoin and anticonvulsant activity after oral administration of phenytoin-bis-hydroxyisobutyrate to rats. *Pharmaceutical Research* **14**, 251-253.
- Seedher, N. and Bhatia, S., 2003. Solubility enhancement of Cox-2 inhibitors using various solvent systems. *AAPS PharmSciTech* **4**(3), Article 33.
- Segota, S. and Tezak, D., 2006. Spontaneous formation of vesicles. *Advances in Colloid and Interface Science* **121**, 51-75.

Serajuddin, A.T.M., 2007. Salt formation to improve drug solubility. *Advanced Drug Delivery Reviews* **59**(7), 603-616.

Sharma, A. and Sharma, U.S., 1997. Liposomes in drug delivery: progress and limitations. *International Journal of Pharmaceutics* **154**, 123-140.

Shieh, M-F., Chu, I-M., Lee, C-J., Kan, P., Hau, D-M., Shieh, J-J., 1997. Liposomal delivery system for taxol. *Journal of Fermentation and Bioengineering* **83**(1), 87-90.

Sigma Aldrich, 2008.
<http://www.sigmaaldrich.com/Area_of_Interest/Europe_Home/UK.html>

Sinha, J., Raay, B., Das, N., Medda, S., Garai, S., Mahota, S.B., Basu, M.K., 2002. Bacopasaponin C: critical evaluation of anti-leishmanial properties in various delivery modes. *Drug Delivery* **9**, 55-62.

Smistad, G., Jacobsen, J., Sande, S.A., 2007. Multivariate toxicity screening of liposomal formulations on a human buccal cell line. *International Journal of Pharmaceutics* **330**, 14-22.

Souza, S.M.B., Oliveira Jr., O.N., Scarpa, M.V., Oliveira, A.G., 2004. Study of the diclofenac/phospholipid interactions with liposomes and monolayers. *Colloids and Surfaces B: Biointerfaces* **36**, 13-17.

Stella, V.J., 1996. A case for prodrugs: fosphenytoin. *Advanced Drug Delivery Reviews* **19**, 311-330.

Stevens, J., Mims, P., Coles, N., 2003. Lipid emulsions as drug delivery systems. *Business Briefing: Pharmatech*, 1-4.

Streng, W.H., Hsi, S.K., Helms, P.E., Tan, H.G., 1984. General treatment of pH-solubility profiles of weak acids and bases and the effects of different salts on the solubility of a weak base, *Journal of Pharmaceutical Sciences* **73**, 1679-1684.

Suzumura, M., 2005. Phospholipids in marine environments: a review. *Talanta* **66**, 422-434.

Talsma, H., Gooris, G., van Steenbergen, M.J., Salomons, M.A., Bouwstra, J., Crommelin, D.J.A., 1992. The influence of the molar ratio of cholesteryl hemisuccinate/dipalmitoylphosphatidylcholine on 'liposome' formation after lipid film hydration. *Chemistry and Physics of Lipids* **62**, 105-112.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1990. Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes. *International Journal of Pharmaceutics* **58**, 49-55.

Terzano, C., Allegra, L., Alhaique, F., Marianecchi, C., Carafa, M., 2005. Non-phospholipid vesicles for pulmonary glucocorticoid delivery. *European Journal of Pharmaceutics and Biopharmaceutics* **59**, 57-62.

Tiberg, F., 2003. Improving drug delivery by use of lipid self-assembly particle structures- beyond liposomes and emulsions. *Business Briefing: Pharma Outsourcing* 2003, 62-65.

Torchilin, V.P., 2001. Structure and design of polymeric surfactant-based drug delivery systems. *Journal of Controlled Release* **73**, 137-172.

Treat, J., Damjanov, N., Zrada, S., Rahman, A., 2001. Liposomal-encapsulated chemotherapy: Preliminary results of a phase I study of a novel liposomal paclitaxel. *Oncology* **15**(5), 44-48.

Uchegbu, I.F. and Florence, A.T., 1995. Non-ionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry. *Advances in Colloid and Interface Science* **58**, 1-55.

Uchegbu, I.F., Gianasi, E., Cociancinch, F., Florence, A.T., Duncan, R., 1996. Niosome encapsulation of a doxorubicin polymer conjugate. *European Journal of Pharmaceutical Sciences* **4** (Supplement), S28.

Uchegbu, I.F. and Vyas, S.P., 1998. Non-ionic surfactant based vesicles (niosomes) in drug delivery. *International Journal of Pharmaceutics* **172**, 33-70.

Uchegbu, I.F., 1999. Parenteral drug delivery: 1. *Pharmaceutical Journal* **263**, 309-318.

Uhrikova, D., Balgavy, P., Kucerka, N., Islamov, A., Gordeliy, V., Kuklin, A., 2000. Small-angle neutron scattering study of the *n*-decane effect on the bilayer thickness in extruded unilamellar dioleoylphosphatidylcholine liposomes. *Biophysical Chemistry* **88**, 165-170.

Vangala, A., Kirby, D., Rosenkrands, I., Agger, E.M., Andersen, P., Perrie, Y., 2006. A comparative study of cationic liposome and niosome-based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice. *Journal of Pharmacy and Pharmacology* **58**, 787-799.

Vanlerberghe, G., Handjani-Vila, R.M., Berthelot, C., Sebag, H., 1973. Synthese et activite de surface compare d'une serie de nouveaux derives non-ioniques. *Proceedings of the 6th International Federation of Cosmetic Chemists*.

Varshosaz, J., Pardakhty, A., Hajhashemi, V-I., 2003. Development and physical characterisation of sorbitan monoester niosomes for insulin oral delivery. *Drug Delivery* **10**, 251-262.

Vilcheze, C., McMullen, T.P.W., McElhaney, R.N., Bittman, R., 1996. The effect of side-chain analogues of cholesterol on the thermotropic phase behaviour of 1-stearoyl-2-oleoylphosphatidylcholine bilayers: a differential scanning calorimetric study. *Biochimica et Biophysica Acta* **1279**, 235-242.

Volodkin, D., Mohwald, H., Voegel, J-C., Ball, V., 2007. Coating of negatively charged liposomes by polylysine: Drug release study. *Journal of Controlled Release* **117**, 111-120.

Vyas, S.P., Kannan, M.E., Jain, S., Mishra, V., Singh, P., 2004. Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. *International Journal of Pharmaceutics* **269**, 37-49.

Wang, Y., Krull, I.S., Carrie, L., Orr, J.D., 2003. Derivatization of phospholipids. *Journal of Chromatography B* **793**, 3-14.

Whelan, M.R., Ford, J.L., Powell, M.W., 2002. Simultaneous determination of ibuprofen and hydroxypropylmethylcellulose (HPMC) using HPLC and evaporative light scattering detection. *Journal of Pharmaceutical and Biomedical Analysis* **30**, 1355-1359.

Wiseman, H., Quinn, P., Halliwell, B., 1993. Tamoxifen and related compounds decrease membrane fluidity in liposomes. Mechanism for the antioxidant action of tamoxifen and relevance to its anticancer and cardioprotective actions? *FEBS Letters* **330**:1, 53-56.

Wishart, D.S., Knox, C., Guo, A.C., Shrivastava, S., Hassanali, M., Stothard, P., Chang, Z., Woolsey, J., 2006. DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic Acids Research*, 2006 Jan 1;34 (Database issue), D668-672.

Xu, G. and Sunada, H., 1995. Influence of formulation change on drug release kinetics from hydroxypropylmethylcellulose matrix tablets. *Chemical & Pharmaceutical Bulletin* **43**(3), 483-487.

Yamauchi, M., Tsutsumi, K., Abe, M., Uosaki, Y., Nakakura, M., Aoki, N., 2007. Release of drugs from liposomes varies with particle size. *Biological & Pharmaceutical Bulletin* **30**(5), 963-966.

Yoshioka, T. and Florence, A.T., 1994. Vesicle (niosome)-in-water-in-oil (v/w/o) emulsions: an in vitro study. *International Journal of Pharmaceutics* **108**, 117-123.

Yoshioka, T., Sternberg, B., Florence, A.T., 1994. Preparation and properties of vesicles (niosomes) of sorbitan monoesters (Span 20, 40, 60 and 80) and a sorbitan triester (Span 85). *International Journal of Pharmaceutics* **105**, 1-6.

Zhang, J.A., Anyarambhatla, G., Ma, L., Ugwu, S., Xuan, T., Sardone, T., Ahmad, I., 2005. Development and characterisation of a novel Cremophor® EL free liposome-based paclitaxel (LEP-ETU) formulation. *European Journal of Pharmaceutics and*

Biopharmaceutics **59**, 177-187.

Zhao, L. and Feng, S-S., 2006. Effects of cholesterol component on molecular interactions between paclitaxel and phospholipid within the lipid monolayer at the air-water interface. *Journal of Colloid and Interface Science* **300**, 314-326.